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14. ABSTRACT

Our basic understanding of how prostate cancer metastasis develops is limited. The recent identification of genes, whose expression suppresses metastasis but not growth in xenograft models, has provided a potential avenue for better understanding the metastatic process. The overall objective of this proposal is to determine how loss of the metastasis suppressor, KAI1/CD82, promotes the development of metastatic prostate cancer. Elevated expression of integrins a6b1 and a3b1 is highly correlative with the invasive and metastatic phenotype of prostate cancer. It has been proposed that migration of tumor cells on laminin-enriched nerve fibers via integrins facilitates prostate cancer spread. The metastasis suppressor KAI1/CD82 is known to associate with a6b1 and a3b1 integrin laminin receptors. We previously demonstrated that adhesion of metastatic prostate cancer cells to laminin induces activation of the metastasis associated receptor tyrosine kinase c-Met, and that re-expression of KAI1/CD82 suppresses both laminin- and HGF-induced c-Met activation. c-Met is up-regulated in all metastatic prostate cancers and is a physiological mediator of cell migration and invasion. Thus, we hypothesize that loss of CD82/KAI1 expression in primary prostate cancer results in enhanced activation of c-Met via both its ligand HGF and laminin integrins, which influences downstream signaling that is required to promote metastasis.

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CD82 Review Paper

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INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer and the third leading cause of cancer deaths in men in the United States (7). Death is due to invasion and metastasis beyond the prostate gland, primarily into the bone and occasionally to other peripheral organs. Our basic understanding of how prostate cancer metastasis develops is limited. The recent identification of genes, whose expression suppresses metastasis but not growth in xenograft models, has provided a potential avenue for better understanding the metastatic process (1). **The overall objective of this proposal is to determine how loss of the metastasis suppressor, KAI1/CD82, promotes the development of metastatic prostate cancer.**

Elevated expression of two integrins $\alpha6\beta1$ and $\alpha3\beta1$ is highly correlative with the invasive and metastatic phenotype of prostate cancer. It has been proposed that migration of tumor cells on laminin-enriched nerve fibers via $\alpha6\beta1$ and $\alpha3\beta1$ integrins facilitates prostate cancer spread (4). The metastasis suppressor KAI1/CD82 is known to associate with $\alpha6\beta1$ and $\alpha3\beta1$ integrin laminin receptors. We previously demonstrated that adhesion of metastatic prostate cancer cells to laminin induces activation of the metastasis associated receptor tyrosine kinase c-Met, and that re-expression of KAI1/CD82 suppresses both laminin- and HGF-induced c-Met activation (8). c-Met is up-regulated in all metastatic prostate cancers and is a physiological mediator of cell migration and invasion (6). **Thus we hypothesize that loss of CD82/KAI1 expression in primary prostate cancer results in enhanced activation of c-Met via both its ligand HGF and laminin integrins, which influences downstream signaling that is required to promote metastasis.**

BODY

Summary of Aim 1. The goal of aim 1 is to identify the mechanism by which CD82 regulates c-Met activity. Our *working hypothesis* is that CD82 negatively regulates c-Met activation through CD82-specific association with integrins and other tetraspanin molecules. Our **first task** is to determine the region on CD82 that interacts with integrins. To accomplish this we first needed to generate mutants of CD82 that we predict would no longer interact with integrins. We initially focused on the second extracellular domain of CD82, a region found in other tetraspanins to be important for a direct integrin interaction (13). In addition to a direct interaction, some tetraspanins interact with integrins via other tetraspanins when they form heterodimers. The formation of tetraspanin heterodimers depends on their palmitoylation (12). Thus we will also investigate this interaction. A third approach we proposed is to generate chimeric molecules between the highly unrelated uroplakin tetraspanin (UPIb) and CD82.

As presented in the original preliminary data of this proposal, we have successfully generated both point mutants and a small deletion mutant in the EC2 domain of CD82. These have all been HA-tagged at the C-terminus and were expressed when transiently transfected into PC3 cells (Fig 1). We have also obtained the palmitoylation-deficient CD82 mutant, which has previously been shown to have reduced affinity for tetraspanins (15). We have not yet generated the CD82/UP1b chimeras, but have obtained the UP1b cDNA (9).

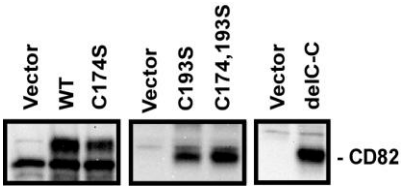


Figure 1: A) PC3 cells were transiently transfected with 4ug of empty vector (Vector), wild type (Wt) and several EC2 mutants of HA-tagged CD82. Forty eight hours later cells were lysed and analyzed by immunoblotting with anti-HA antibody.

We have begun generating stable PC3 cell line expressing the CD82 mutants. So far we have been able to successfully establish several clones of PC3 cells expressing the

palmitoylation deficient mutant, the EC2 domain deletion, as well as one of the EC2 domain point mutants (Fig 2). The other point mutant constructs do not appear to be expressed well in stable cell lines – likely due to protein destabilization.

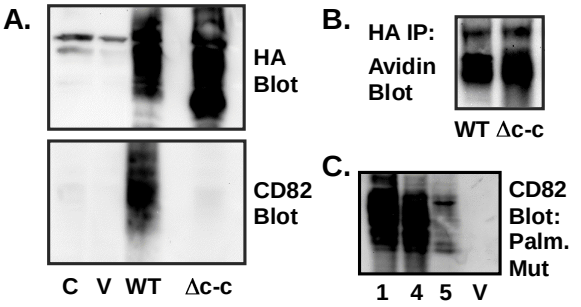
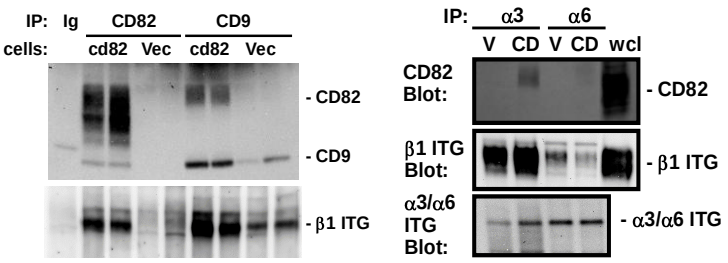


Figure 2: A) Cell extracts from PC3 cells stably expressing vector (V), wild type HA-tagged CD82 (WT), or the deletion mutant (Δc-c) or **C)** palmitoylation mutant were analyzed for CD82 expression by immunoblotting with CD82 antibody (CD82 blot) or HA antibody (HA Blot). As expected, mutation of the EC2 domain abolishes binding of the EC2 domain epitope antibody, but expression is still detectable by HA antibody. The broad band is indicative of successful post-translational modification. **B)** Both wild type CD82 and the deletion mutant were detectable on the cell surface as measured by biotin surface labeling of stable PC3 cell lines.

The next step is to determine whether the CD82 mutants are still able to associate with $\alpha 6\beta 1$ and $\alpha 3\beta 1$ integrins or other tetraspanins. Preliminary experiments aimed at investigating whether wild type CD82 can associate with integrins or other tetraspanins revealed that CD82 could be co-immunopreciptated with tetraspanins CD9 and CD151, as well as $\alpha 3\beta 1$ integrin (Fig 3). There was a weak association of CD82 with $\alpha 6\beta 1$ integrin. We have not yet tested the mutants. However, the palmitoylation mutant was previously shown to have reduced association with tetraspanins, but still retained its association with $\alpha 3\beta 1$ integrin (15).

Figure 3: A) CD82 or CD9 were immuno-precipitated (IP) from adherent vector-transfected (Vec) or CD82 expressing (CD82) PC3 cells in duplicate. Immunoprecipitates (IP) were monitored for the presence of CD9, CD82 or β1 integrin (β1 ITG) by immune-blotting. Mouse immunoglobulin (Ig) was used as a negative IP control. **B)** α3 or α6 laminin integrins were immunoprecipitated (IP) from vector (V) or CD82 expressing cells (CD) and the levels of CD82 (CD82) or β1 integrin (β1 ITG) were monitored by immunoblotting



Our **second task** is to identify the region on CD82 that is responsible for suppressing c-Met activity. The same mutants that were generated above will be tested for their ability to suppress c-Met activity. Thus far we have tested only the EC2 domain deletion mutant, and found that it failed to suppress c-Met activity (Fig 4).

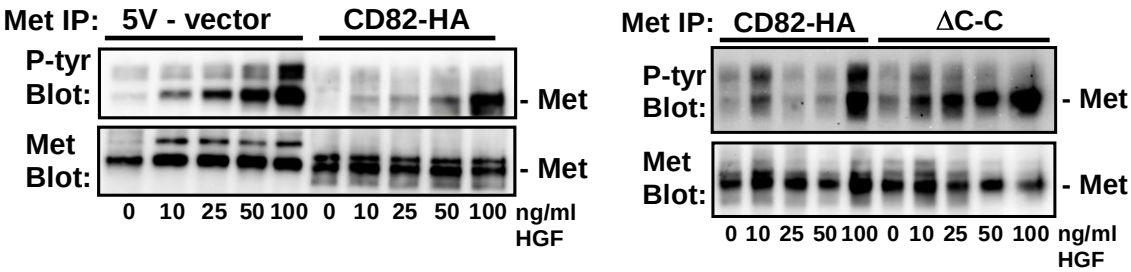


Figure 4: PC3 cells expressing vector (5V), wild type HA-tagged CD82 (CD82-HA), or the HA-tagged deletion mutant (Δc-c) were stimulated with increasing concentrations of HGF. C-Met activation was measured by immunoblotting immunoprecipitates with anti-phosphotyrosine antibodies. CD82 suppresses the c-Met activation at low doses of HGF, but has no effect on high doses of HGF. The deletion mutant was no longer able to suppress c-Met activation at low doses of HGF.

One of the models we have proposed is that CD82 association with integrins via other tetraspanins is responsible for c-Met suppression. If this is true, then the removal of other tetraspanins, i.e. those that associate with CD82, would block the ability of CD82 to suppress c-Met. To test this hypothesis, we generated siRNA sequences to CD9 and CD151, both of which associate with CD82. Transfection of PC3 cells expressing wild type CD82 with CD9 specific siRNA, but not scrambled siRNA or CD151 siRNA (not shown), restored c-Met activation (Fig 5). Furthermore, loss of CD9, but not CD151 (not shown), also restored matrigel invasion in CD82-expressing cells (Fig 6). Thus, CD9, but not CD151, is required for CD82 to effectively suppress c-Met activity and invasiveness.

Figure 5: PC3 cells expressing CD82 were transfected with CD9 siRNA or a scrambled (scrm) sequence and then stimulated with increasing concentrations of HGF. The level of c-Met activation was measured by immunoblotting of immuno-precipitates with anti-phosphotyrosine antibody (P-tyr Blot). The levels of CD9 and CD82 were monitored by blotting.

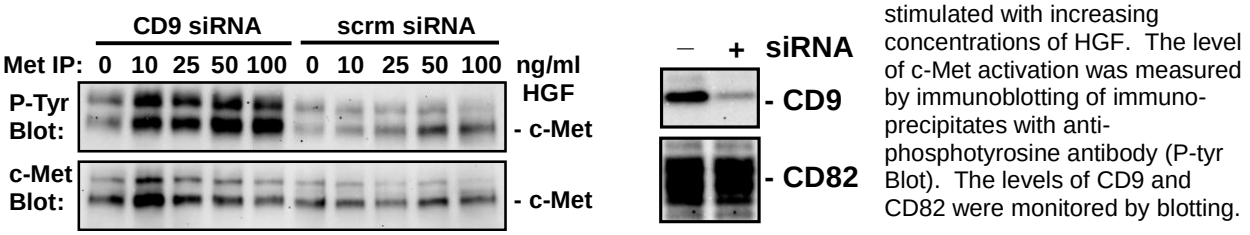
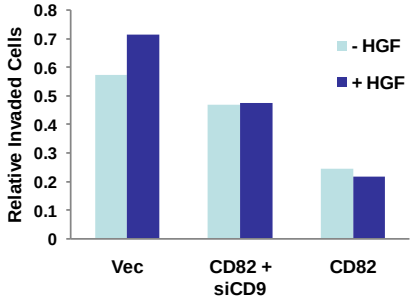


Figure 6: PC3 cells expressing CD82 were transfected with CD9 siRNA or a scrambled sequence (CD82) and then allowed to invade matrigel. The ability to invade through the matrigel was quantified by reading the OD of the dye released from the stained cells that successfully invaded through the matrigel. CD82 expression suppresses invasion compared to non-CD82 expressing cells (Vec), but requires CD9 to effectively suppress invasion.



Our **third task** is to determine if CD82 interaction with integrins is responsible for suppressing c-Met activity. We have not begun these studies yet.

Summary of Aim 2. The goal of aim 2 is to determine how loss of CD82 leads to metastatic prostate cancer. Our *working hypothesis* is that CD82 loss *in vivo* results in increased c-Met signaling, both of which are required for the development of metastatic disease. Our **first task** is to determine if CD82 expression inhibits metastasis in HGF transgenic mice. We have demonstrated that DU145 cells will only invade matrigel in the presence of HGF and CD82 suppresses this HGF-dependent invasion [Sridhar, 2006], suggesting that inhibition of invasion is mediated by suppression of c-Met. We wished to test this in an in vivo xenograft model. Only human HGF will bind human c-Met, thus we have taken advantage of transgenic SCID mice which over express human HGF (14). In this model HGF interaction with c-Met on the metastatic prostate cancer cell line DU145 was predicted to induce HGF/c-Met-dependent metastasis. Indeed we found this to be the case. Orthotopic injection of metastatic DU145 cells failed to generate metastases in normal SCID mice, while inducing metastasis in 60-95% of the HGF-SCID mice (Table 1). CD82 expression suppressed metastasis, but not growth.

TABLE 1: DU145 tumorigenesis and metastasis in SCID and HGF/SCID mice

Mice	Tumors	Metastases
SCID	31/36 (86%)	0/31 (0%)
SCID-HGF	31/36 (86%)	18/31 (58%)

SCID+HGF	Tumors	Metastases
- CD82	20/20 (100%)	19/20 (95%)
+ CD82	20/20 (100%)	1/20 (5%)*

* CD82 expression was lost in this single lymph node metastasis

Tumor samples were isolated and immunostained for CD82 expression, active c-Met (using a phospho-specific antibody), and total c-Met. While c-Met was present in the CD82-expressing tumors, it was not active, whereas it was active in the tumors not expressing CD82 (Fig 7). Thus HGF-/c-Met dependent metastasis of DU145 cells is inhibited by expression of CD82, and is accompanied by a loss in c-Met activation in vivo. The mutants being generated in Aim 1 will be used to determine if they still have the capacity to suppress metastasis and whether that is associated with a loss in c-Met activation.

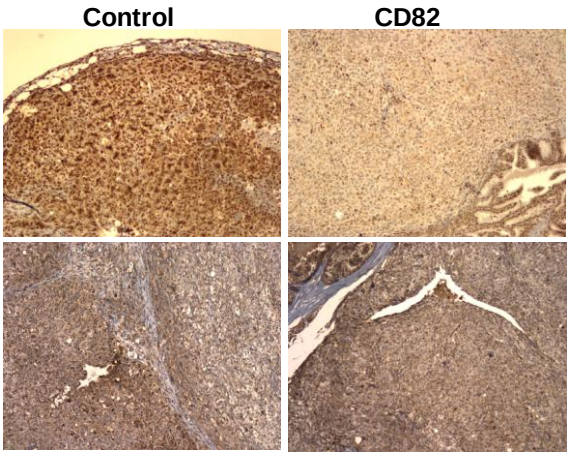


Figure 7: Primary prostate tumors isolated from HGF/SCID mice following orthotopic injection of parental DU145 cells (control) or CD82-expressing cells (CD82) were immunostained for active c-Met (P-MET) and total c-Met (Total MET).

Our **second task** is to determine if loss of CD82 expression in mice genetically manipulated to produce prostate tumors is required for the development of metastatic disease. We have generated floxed CD82 mice. These mice were crossed to CMV-CRE mice to generate a complete knock-out of CD82 or to Probasin-Cre mice to generate conditional loss of CD82 in prostate epithelial cells. RT-PCR analysis of either whole prostates or laser captured prostate epithelial cells indicate that Cre-induced recombination in the prostate gland generates a shorter mRNA, missing the expected internal exon sequences (Fig 8).

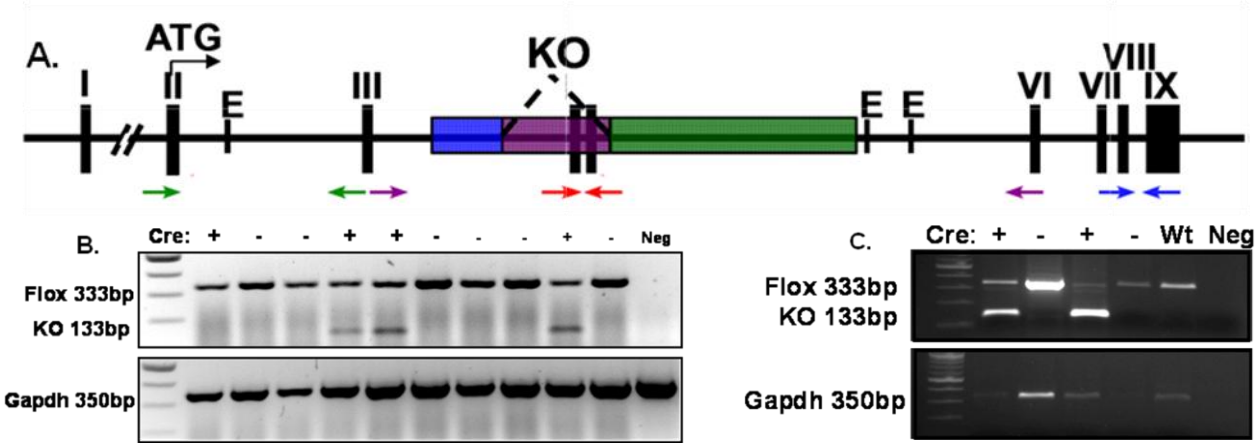


Figure 8: **A)** Cre-mediated loss of Exons IV and V (purple region) would be expected to generate a shortened mRNA product (purple arrows) across exons III to VI. **B)** RT-PCR across exons III and VI of RNA isolated from whole prostate glands of Cre(+) and non Cre(-) expressing mice 16 weeks of age. **C)** RT-PCR across exons III to VI of RNA isolated from prostate epithelial cells after laser capture from 20 week old Cre(+) and Cre(-) mice.

RT-PCR analyses of CMV-Cre/CD82 null mice indicate the same change in mRNA size in other tissues (kidney, liver, spleen) as observed in prostate tissue (data not shown). Protein expression analysis of CD82 in various mouse tissues from the CD82 null mice derived from CMV-Cre recombination indicated complete loss of CD82 expression (Fig 9). In addition, there were no changes in the expression of other tetraspanin genes in these mice (data not shown).

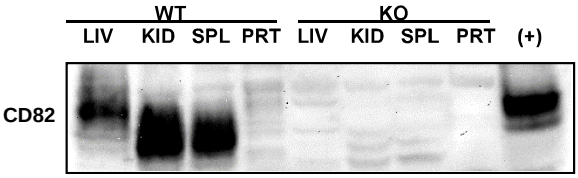


Figure 9: Livers (LIV), kidneys (KID), spleens (SPL) and prostates (PRT) from wild type mice (WT) or CD82 null mice (KO) were harvested and tissue extracts were analyzed by immnoblotting for expression of CD82.

No tumors, no PIN, nor any consistent changes in the prostate epithelium was observed in the prostates of CD82 null mice. Thus, loss of CD82 in the prostate has no effect on normal prostate physiology. The CD82/Probasin-Cre mice have been crossed to mice deficient in Pten expression in the prostate. The oldest $CD82^{pr-/-}$ x $Pten^{pr-/-}$ mice, now 26 weeks old, have begun to develop adenocarcinoma lesions. So far no metastatic lesions have been observed. Mice will continue to be monitored. Genetic background can impact the outcome of tumor studies, and other tetraspanin mice develop distinctly different phenotypes dependent on background. Therefore, we have also backcrossed all three strains, $CD82^{flox/flox}$, Probasin-Cre, and $Pten^{flox/flox}$ mice, into both FVB/N and Balb/c backgrounds. The FVB/N backcrosses have recently been completed (assisted by speed congenic strain analysis), and the Balb/c backcrosses are still in progress. Crossing ofl the FVB/N strains together has begun.

Preliminary studies in the CD82 null mice indicate that there are alterations in endothelial-mediated angiogenesis, i.e. these mice have an increased capacity to initiate new blood vessel formation. There is also a likely defect in platelet function – these mice do not bleed as readily as wild type mice. These phenotypes are the exact opposite of those observed in CD151 null mice, suggesting a reciprocal phenotypic relationship between CD151 and CD82. Whether this relationship is important for metastasis has yet to be determined.

KEY RESEARCH ACCOMPLISHMENTS

1. Generated stable transfectants of PC3 cells expressing several mutants of CD82.
2. Demonstrated that the EC2 domain of CD82 is required for CD82-mediated suppression of c-Met activity.
3. Demonstrated that CD82 preferentially associates with $\alpha 3\beta 1$ integrin, CD9, and CD151.
4. Demonstrated that CD9 is required for CD82-mediated suppression of c-Met activity and suppression of matrigel invasion.
5. Demonstrated that expression of CD82 in DU145 cells completely suppresses HGF/c-Met-dependent metastasis, which is accompanied by a complete loss of c-Met activation in the primary tumors.
6. Successfully generated CD82 null mice as well as mice in which CD82 expression is absent in prostate epithelial cells.
7. Generated FVB/N specific strains of $CD82^{flox/flox}$, Probasin-Cre, and $Pten^{flox/flox}$ mice.

REPORTABLE OUTCOMES

The following items have been generated due to the research carried out in the last year.

1. We have published an invited review paper on the role of CD82 in metastasis. A copy is in the appendix.

> **Miranti, C.K.** 2009. Controlling cell surface dynamics and signaling: how CD82/KAI1 suppresses metastasis. **Cellular Signaling** 21:196–211.

2. One abstract was delivered as an oral presentation at a scientific meeting. The abstract is in the appendix.

Presented by Dr. Miranti:

Miranti, C.K. 2008. CD82 Suppresses Metastasis via Inhibition of c-Met. **FASEB: Signal Transduction Through Tetraspanins and Other Multi-Protein Cell Surface Complexes**, New Haven, Connecticut, June 22-29.

3. We have generated stable cell lines of PC3 cells expressing mutants of CD82. These will be useful for others interested in studying CD82 function.

4. We have developed an HGF/c-Met-dependent metastasis model using DU145 cells and HGF/SCID mice. This model will be valuable for assessing c-Met-dependent prostate tumors and metastasis, and potentially as a preclinical drug screening model.

5. We have generated the first conditional CD82 knock-out mouse. This model will be useful for analyzing the role of CD82 in many biological/organ processes, useful for distinguishing cell origin of specific phenotypes, and can be used to assess the role of CD82 suppression of metastasis in any primary cancer model.

6. We are the first lab to generate $CD8^{flox/flox}$, Probasin-Cre, and $Pten^{flox/flox}$ strains in a homogeneous FVB/N background. These strains will be immensely valuable in assessing the effects of genetic background on prostate cancer susceptibility and progression.

CONCLUSIONS

Prior to our studies the role of CD82 loss in regulating prostate tumor metastasis had not been determined. We have demonstrated that in tumor cells where c-Met expression is responsible for enhancing migration and invasion in vitro, re-expression of CD82 suppresses c-Met function. We have also shown this to be true in vivo. We have generated mutants of CD82 that will allow us to access the relationship between CD82 loss and c-Met activation in vitro and in vivo. Our studies will also allow us to determine which of the many functions attributed to CD82 in vitro are required for its metastatic suppressive activity in vivo.

So What: Our findings have broad implications for the control of metastatic cancer. CD82 loss has been reported in many types of cancers. Likewise, c-Met over expression, mutation, or activation has also been reported for a wide range of cancers and its aberrant activity correlates with the development of metastasis (2, 3, 10). We propose that loss of CD82 may be required for the development of metastasis, by removing a control point for c-Met signaling. These studies will establish whether this is true in an in vivo setting. If this proves to be so, then the mouse models that we have generated in these studies will serve as excellent preclinical models for drug testing, and defining more precisely the molecular mechanisms involved.

Our studies will also advance the knowledge of how members of the tetraspanin family function. Many possible functions have been attributed to CD82, but it is not clear which ones are relevant to its metastasis suppressor functions. Interestingly, two other tetraspanins, CD151 and CO-029, appear to behave opposite to CD82, in that their levels of expression and activity are elevated in tumors (5, 11). Since tetraspanins are known to interact with each other, it is possible that loss of CD82 may act in part by enhancing the expression or activity of other tetraspanins to drive metastasis. Our studies will determine if this is a possible mechanism.

PERSONNEL SUPPORTED BY GRANT

Dr. Cynthia Miranti	20% effort
Kristen Saari	100% effort
Susan Spotts	50% effort

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APPENDIX

Meeting Abstract

CD82 Suppresses Metastasis via Inhibition of c-Met

Cindy K. Miranti

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Loss of CD82 expression in prostate cancer correlates with progression to metastasis. We have demonstrated that re-expression of CD82 at physiological levels in metastatic prostate tumor cell lines, suppresses integrin- or HGF-mediated activation of the receptor tyrosine kinase c-Met. Signaling through c-Met is required for cell migration and invasion in metastatic prostate tumor cells and is over expressed in all metastatic prostate cancers. We are investigating the role of CD82 in suppressing c-Met in vivo and determining the mechanism by which CD82 suppresses c-Met activity. Orthotopic injection of DU145 cells into SCID mice over expressing human-specific HGF results in 100% induction of prostate cancer metastases, compared to 0% metastases after injection into normal SCID mice. CD82 re-expression in DU145 cells suppresses prostate cancer metastasis by 95%, but has no effect on primary tumor growth. Activated c-Met could be detected in primary and metastatic tumors from HGF-SCID mice, while activated c-Met was not detected in primary tumors from CD82-expressing cells. We have successfully generated CD82 null mice, which appear normal and are fertile. The CD82 mice are being crossed with Pten conditional mice, which develop primary but not metastatic prostate cancer, to determine if loss of CD82 is sufficient to induce prostate cancer metastasis in Pten null mouse prostates. Coimmunoprecipitation experiments have failed to detect a direct interaction between CD82 and c-Met, thus the effect of CD82 on c-Met must be indirect. CD82 is known to associate with integrins and integrins can control c-Met activation. CD82 was found to associate with $\alpha 3\beta 1$ integrin and CD9 in prostate cells. siRNAs directed to CD9 blocked the ability of CD82 to suppress c-Met activation. A small deletion in the EC2 domain, but not inhibition of palmitoylation, generated a CD82 mutant that failed to inhibit c-Met activation. These data indicate that the extracellular domain of CD82 and CD9 are required to suppress c-Met activation.

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Review

Controlling cell surface dynamics and signaling: How CD82/KAI1 suppresses metastasis

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ABSTRACT

The recent identification of metastasis suppressor genes, uniquely responsible for negatively controlling cancer metastasis, are providing inroads into the molecular machinery involved in metastasis. While the normal function of a few of these genes is known; the molecular events associated with their loss that promotes tumor metastasis is largely not understood. KAI1/CD82, whose loss is associated with a wide variety of metastatic cancers, belongs to the tetraspanin family. Despite intense scrutiny, many aspects of how CD82 specifically functions as a metastasis suppressor and its role in normal biology remain to be determined. This review will focus on the molecular events associated with CD82 loss, the potential impact on signaling pathways that regulate cellular processes associated with metastasis, and its relationship with other metastasis suppressor genes.

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1. Introduction

Metastatic cancer remains an incurable disease. The successful identification and analysis of tumor suppressor genes responsible for the initiation of primary tumors, and identification of the genes that they suppress, has been key to the successful development of new cancer therapies. It is logical to propose that a similar approach, i.e. identification of metastasis suppressor genes, would be equally beneficial to curing metastatic disease. Indeed, such genes exist; over 20 metastasis suppressor genes have been identified based on their specific ability to suppress metastasis, but not primary tumor growth, in xenograft models [1,2]. Many follow-up studies in human cancer tissues support their role as metastasis suppressors, as loss of expression is observed almost exclusively in metastatic disease. The normal biological function of several, but not all, metastasis genes are known; however, few have been characterized with respect to how their loss promotes tumor metastasis. It is not even known if loss of any of these genes in vivo is sufficient or absolutely required for metastasis. This review will focus on what is known about the metastasis suppressor gene, KAI1/CD82. Despite intense scrutiny, many aspects of how CD82 specifically functions as a metastasis suppressor and its role in normal biology remain to be determined.

1.1. Establishment of KAI1 (CD82) as a metastasis suppressor

The metastasis suppressor function of KAI1/CD82 was first detected in a genetic screen using the metastatic rat AT6.1 prostate cancer cell line expressing fragments of chromosome 11, on which CD82 is located [3]. Re-expression of CD82 in AT6.1 cells and subsequent subcutaneous injection into nude mice significantly reduced metastases without affecting primary tumor growth. Numerous xenograft studies using other metastatic cell lines, including MDA-MB-435, breast LCC6, liver MHCC97-H, lung LLC, HT1080 sarcoma, and prostate LNCaP, further confirmed the metastasis suppressor function of CD82 [4–9]. The route of injection, subcutaneous, orthotopic, or tail vein, did not impact the outcome and no major effects on primary tumor growth were seen in any model.

That CD82 is a valid metastasis suppressor gene is further supported by numerous clinical studies. Loss of CD82, both protein and mRNA, is strongly correlated with poor prognosis in many malignancies (reviewed in [10]), including prostate, colon, lung, pancreatic, breast, ovarian, and several others. While the direct association between CD82 and metastasis per se is not always straight forward, the number of reports citing CD82 loss out-numbers five to one those reporting an increase or no change. In two prostate cancer mouse models, down regulation of CD82 expression was observed in metastatic disease (CKM unpublished data)[11]. Thus CD82 loss is highly correlative with metastatic disease, CD82 loss occurs at the transcriptional level, and based on xenograft animal models CD82 suppresses metastasis. Furthermore, CD82 loss is likely to be a universal event in the development of metastasis since it is observed in many types of cancer.

1.2. CD82 molecular biology

CD82 was first cloned based on its affinity for several monoclonal antibodies (R2, IA4, C33, 4F9) that recognize lymphocytic surface antigens [12–16]. CD82 was subsequently assigned membership in the Cluster of Differentiation antigens [17]. CD82 is a member of the 4-span transmembrane super family (TM4SF) of type III membrane proteins, specifically of the tetraspanin subgroup (Tspan). There are 33 tetraspanins in the human genome (Table 1). Tetraspanin proteins are not present in yeast or bacteria, but are present in fungi and all multicellular organisms [18]. Direct functional comparisons between divergent species are difficult because of low conserved DNA sequence homology.

CD82 and several other tetraspanins are fairly ubiquitously expressed. Northern analysis of human tissues reveals high expression of CD82 in the spleen, thymus, prostate, ovary, small intestine, colon,

Table 1
Tetraspanin family genes, tissues expressed, and identified functions

Tspan	Alt. names	Tissues	Function
1	NET-1	Liver cancer	Tumorigenesis
2	?	CNS	Oligodendrocyte differentiation
3	OAP-1, TM4SF8	Oligodendrocytes	Migration, proliferation
4	NAG-2, TM4SF7	Fibroblasts	Unkn
5	NET-4, TM4SF9	CNS	Unkn
		Osteoclasts	Osteoclastogenesis
6	TM4SF6	?	X-linked gene
7	TM4SF2, A15	Neurons	Mental retardation
8	CO-029, D6.1A	Colon cancer	Metastasis, angiogenesis
9	NET-5	Ovarian cancer	Amplified 12p gene
10	OCSP, oculospanin	Pigment epithelium	?
11	?	?	?
12	NET-2	?	?
13	NET-6	Breast cancer	Tumor suppressor
		Osteoclasts	Osteoclastogenesis
14	?, TM4SF14	?	?
15	NET-7	?	?
16	TM-8, TM4-B	?	?
17	?, TM4SF17	?	?
18	Neurospanin	Brain, CNS	?
19	?	?	?
20	Uroplakin1b	Bladder	Urine permeability barrier
21	Uroplakin1a	Bladder	Urine permeability barrier
22	RDS/peripherin	Rods/cones	Retinal structure
23	ROM1	Rods	Survival of photoreceptors
24	CD151	Endothelial cells	Angiogenesis
		Platelets	Thrombi stabilization
		Kidney	Organ structure
		Epithelial	Wound healing
25	CD53/OX44	Immune cells	B-cell signaling
		Neutrophils	Immune surveillance
26	CD37	Immune cells	T/B-cell signaling
27	CD82	Epithelial cells	Tumor metastasis suppressor
		Immune cells	T-cell signaling
28	CD81	Oocytes	Egg/sperm fusion
		Immune cells	B-cell signaling
		Brain	Astrocyte/glia proliferation
		Pigment pithelia	Proliferation
29	CD9	Oocytes	Egg/sperm fusion
		Immune cells	T/B-cell function
		PNS	Myelination
30	CD63	Ubiquitous	Protein trafficking
31	SAS	Sarcoma cells	Sarcoma amplified sequence
32	TSSC6	Immune cells	T-cell proliferation
		Platelets	Thrombi stabilization
33	Penumbra	Erythroblasts	Erythropoiesis

placenta, lung, liver, kidney, and pancreas. Significantly, lower expression is seen in the heart, brain, muscle, and testis [3]. Mouse CD82 mRNA was highest in the spleen, kidney, lung, and liver [19], with a similar distribution seen in the rat [20]. Immunostaining of mouse tissues reveals distinct subtissue distributions. Mouse CD82 protein is primarily localized to spleen lymphoid tissue, medullary collecting ducts and distal convoluted tubules of the kidney, arteriolar smooth muscle of the lung, hepatocytes and sinusoidal lining of the liver, islet cells, and is found in many epithelial cells including epididymus, prostate; colon; bladder, ureter, urethra, uterus, ovary, oviducts, testes, and seminal vesicles. CD82 was also present in most vascular endothelium except arterioles and the brain [21].

1.2.1. Transcriptional regulation

There is little evidence for gene mutation, loss of heterozygosity, promoter mutation, or hypermethylation to explain the loss of CD82 expression in clinical isolates of metastatic cancers [22–28]. Altered transcription or splice variants remain as possible mechanisms for loss of CD82 mRNA. One splice variant in which exon 7 is deleted has been reported [29]. Spliced KAI1 mRNA was detected in metastatic and invasive tissues of gastric and bladder cancers as well as several cell lines [29,30]. However, the level of spliced transcript was present at several-fold lower levels than full length mRNA, and did not correlate well with invasiveness or metastasis. Thus its significance in the etiology of metastasis remains unknown.

The human CD82 promoter is G-C rich and Tata-less, and contains an array of potential promoter elements including Sp1, AP-2, GATA-1, PEA3, NF-IL6, MEP1, Myb, TCF-1, HNF3, NF-1, zeste, and Ets binding sites [31,32]. The mouse promoter contains many of the same putative promoter elements [19]. Several extracellular stimuli have been reported to enhance CD82 expression and include cytokines (IL-1β, IL-4, IL-6, IL-13, IFN-γ, TNF-α), growth factors (NGF), phorbol esters (PMA), drugs (Genistein, etoposide), and 8-bromo-cAMP [11,33–39]. The mechanisms by which these stimuli regulate CD82 transcription are virtually unknown, except that NF-κB is known to mediate some of the effects of the cytokines.

Promoter deletion analysis initially identified three transcriptional regions in the CD82 promoter; an enhancer region (−922 to −846); a negative regulatory region (−735 to −197); and the minimal promoter (−197 to +351) [10,32,40]. A p53-like regulatory element, responsible for etoposide induction of CD82, is located at −860 in the enhancer region and was initially intriguing given that loss of p53 in prostate cancer is a late event correlating with progression to metastasis. Despite the fact that over expressed p53 can bind to the promoter and enhance CD82 expression in transfected cells, the correlation between p53 loss and CD82 loss in clinical samples does not stand up, arguing against a strict one-to-one relationship [36,41,42]. Other reports suggest that various combinations of p53, AP2, or JunB binding at the extended AP2-p53-AP1 element are responsible for regulating full CD82 expression and binding of these factors may be differentially altered during metastasis [40,43].

Cytokine induced CD82 expression in immune cells is mediated primarily via NF-κB [44]. In two p53 mutant epithelial cell lines, TNFα-induced CD82 expression was also dependent on NF-κB [45]. Subsequently, ChIP analysis revealed that NF-κB p50, but not p65, is present on the CD82 promoter. The CD82 promoter recruits NF-κB p50, Bcl3 (functionally related to IκBα) and the N-CoR/TAB2/HDAC3 corepressor complex, which results in transcriptional inactivation. IL-1β stimulation transiently recruits Tip60 to p50 bound at the CD82 promoter, which is coincident with loss of the N-CoR complex, increased acetylation and phosphorylation of histones, and recruitment of Pol II. Over expression of a Tip60/Fe65/APP complex was sufficient to displace the N-Cor complex [46]. The Fe65 transcription activation domain binds to the nucleosome assembly factor SET, which is required for Fe65-mediated transactivation. ChIP experiments demonstrated that a complex including Fe65/APP/Tip60 and SET is associated with the CD82 promoter. SET is required for full levels of CD82 transcription.

However, the Tip60 coactivator complex was not recruited to the CD82 promoter in metastatic prostate cancer cells due to low levels of Tip60 expression in these cells [8]. IL-1β-induced Tip60 expression and recruitment could be restored by inhibiting β-catenin expression. A reptin/β-catenin complex was detected at the CD82 promoter that was present only in metastatic cells. The reptin/β-catenin complex could be displaced after IL-1β stimulation by over expressing Tip60. Modulation of Tip60 or β-catenin levels in metastatic cells or normal cells respectively alters IL-β1-mediated matrigel invasion. It was proposed that high levels of the β-catenin–reptin complex, due to Wnt activation, and simultaneous down regulation of Tip60 act together to inhibit CD82 expression and drive metastasis. Subsequently it was shown that the reptin repressive function requires its sumoylation at Lys456 by SENP1/SUSP1 [47]. Blocking reptin sumoylation in metastatic prostate cells restored CD82 mRNA and decreased matrigel invasion.

A recent study in metastatic breast cancer cells suggests CD82 transcription can also be regulated at the level of genomic organization. SATB1 regulates gene expression by recruiting chromatin remodeling enzymes and transcription factors, and tethers multiple genomic loci via specialized DNA sequences to globally control transcription. RNAi-mediated knockdown of SATB1 in metastatic MDA-MB-231 breast cancer cells restored acinar polarity and inhibited tumor growth and metastasis in vivo. Intriguingly, CD82, in addition to several other known metastasis suppressor genes, including nm23, KiSS1, BRMS1, claudin 1, and E-cadherin, were coordinately up-regulated in the SATB1 deleted tumor cells [48]. Thus, loss of p53, enhanced Wnt/β-catenin signaling, stress activated Jnk, and increased expression of SATB1 could all work together or in various combinations to promote the loss of CD82 expression and metastasis.

1.2.2. Post-transcriptional regulation

The incomplete correlation between CD82 mRNA or protein loss and metastasis in some clinical specimens may reflect additional mechanisms for removing CD82 function. Inhibition of E3 ubiquitin ligase gp78 expression in highly metastatic HT1080 sarcoma cells suppressed metastasis, but had no effect on primary tumor growth [9]; an effect that mimics metastasis suppressor genes. CD82 was identified as a primary substrate of gp78. The E3 ligase activity of gp78 was required for its metastatic effects. Loss of gp78 resulted in increased CD82 expression and over expression of gp78 increased CD82 degradation. An inverse relationship between gp78 and CD82 expression was detected in human sarcoma tissue samples and inhibition of CD82 expression in gp78 negative cells restored metastasis. Thus, another possible mechanism for CD82 loss in metastatic tumors is its enhanced degradation or turnover.

Whether other post-transcriptional or translational mechanisms can account for loss of CD82 function in metastatic tumors remains to be addressed. At least two types of post-translational modifications, glycosylation and palmitoylation, are known to occur on CD82. Inhibition of these modifications affects CD82 function [49,50]. Whether the enzymes responsible for CD82 modification are altered in metastatic tumors is not known.

2. CD82 function

Tetraspanin proteins function in many aspects of cell physiology. Several excellent reviews provide extensive information on the genetics, structure, and function of tetraspanins [51–54]. Tetraspanins contain no intrinsic catalytic activity; therefore, current hypotheses favor a model whereby tetraspanins serve as master regulators of membrane organization, through interactions with surface molecules and each other. Through these interactions tetraspanins regulate a variety of cellular events including signaling, transcription, cell

adhesion, migration, survival, endo- and exocytosis, differentiation, and cell fusion. CD82 has been shown to be important for some these cellular processes, but specifically which ones are directly relevant to its metastasis suppressing ability has not been fully determined.

2.1. CD82 structure

The initial characterization of CD82 was aided by its sequence homology to several other tetraspanin proteins, namely CD81, CD9, CD63, and CD37 [12,14,15]. The assignment of CD82 to the tetraspanin family is based on the conserved structural motifs within this family. CD82 is a 267 amino acid type III membrane protein that spans the membrane 4 times, contains short N- and C-terminal cytoplasmic domains, a short 4 amino acid intracellular loop (IC), and two extracellular loops (EC1 and EC2). There is over 90% sequence conservation between human and mouse CD82 proteins within the 4 transmembrane domains, 81% in the cytoplasmic domains, but only 65% in EC2 [14,19]. Within the EC2 domain are three conserved sequence motifs, CCG, PXXC/PCXC, and a GC residue close to the 4th transmembrane domain. These motifs specifically distinguish tetraspanins from other TM4SF proteins [52,53]. The EC2 cysteine residues are predicted to form 2, 3, or 4 disulfide bonds, and are the basis for subclassification within the tetraspanin family [18,55].

Only the EC2 domain of CD81 has been crystallized [56]. Based on CD81, the EC2 structure of other tetraspanins including CD82, as well as the full length protein, were modeled [55,57–59]. The picture of CD82 that emerges is a short cytoplasmic 10 amino acid N-terminal amphipathic helix that lies parallel to the cell membrane and a relatively unstructured 14 amino acid C-terminal tail. The 4 transmembrane domains are tightly packed left-handed antiparallel coiled coils. The 17 amino acid EC1 domain has a hydrophobic beta strand that nestles within a hydrophobic pocket of the EC2 domain. The EC2 domain has two alpha-helical bundles that extend directly up from the two transmembrane domains like a stalk, which supports a third helical domain as well as a highly variable region that is unique to each tetraspanin. CD82 EC2 domain is predicted to have three disulfide bonds within this variable region. The third helix lies parallel to the membrane causing the variable region to sit tilted relative to the stalk. The result is a tightly packed transmembrane protein whose prominent feature is an asymmetric EC2 domain protruding about 5 nm above the cell membrane. The cryo-EM structure of the bladder-specific tetraspanins, uroplakins, also support the notion of a rod like structure extending through the membrane with a prominent binding surface suspended above the membrane [60]. In the uroplakin complex, each tetraspanin protein is tightly associated with its single-pass transmembrane partner, and primarily associate with each other through interactions between the partner molecules, rather than the tetraspanins themselves.

2.2. CD82 in immune signaling

2.2.1. T-cells

The first defined function for CD82 was it role in regulating T-cell signaling through the T-cell receptor (TCR). TCR stimulation activates the src kinases, Fyn and Lck, following recognition of MHC-peptide complexes on antigen presenting cells. Lck and Fyn phosphorylate CD3, leading to recruitment and activation of ZAP-70. Subsequently, ZAP-70 phosphorylates adaptor molecules LAT, SLP-76, and Vav resulting in activation of PKC, MAPK, and Rho GTPases and ultimately transcription of genes such as IL-2 [61]. TCR signaling is maximal in the presence of costimulatory receptors. Monoclonal antibody binding to CD82 can serve as a costimulatory signal for full activation of T cells, and results in strong IL-2 and IFN-γ production and cell differentiation [62]. Costimulation of CD82 also induces cell adhesion, spreading, and actin polymerization [62,63]. Immobilization of CD82 with monoclonal antibody was sufficient to induce tyrosine phosphorylation and association of Vav1 with SLP76, which activates Rho GTPases. Global inactivation of Rho GTPases reduced CD82-induced cell spreading [64]. CD82 can be detected in a complex with TCR [65]. Both the cytoplasmic and extracellular regions of TCR-CD4 were required for CD82 interaction. CD82 initially colocalizes with TCR upon T-cell activation, but relocalizes with F-actin at the periphery of the immune synapse [66] and binding of Lck to TCR precludes CD82 binding [65]. Thus CD82, is involved in dynamic interactions during T-cell signaling.

The T-cell signaling data suggest that the primary function of CD82 in the costimulatory pathway is to facilitate events associated with actin polymerization, a function that is also attributed to integrins. Coincidently, CD82 associates with α4β1 integrin in T-cells [67]. CD82 levels on resting T-cells are low and during TCR activation there is a 3.5- to 7-fold increase in CD82 surface levels [37]. Increased CD82 expression is associated with enhanced homotypic interactions between T-cells [39]. Enhanced adhesion between T-cells was not due to CD82 directly, but was mediated by interactions between ICAM-1 and its integrin receptor LFA-1(αLβ2). CD82 and LFA-1 colocalized at cell–cell interaction points and could be coimmunoprecipitated from T-cells. LFA-1 antibodies could substitute for the CD82 costimulatory signal [68]. Thus the function of CD82 is to facilitate integrin-dependent events in T-cell signaling. Antibodies to other tetraspanins, CD9, CD53 or CD81, have similar costimulatory activity as CD82 antibodies; these tetraspanins also interact with integrins [69].

2.2.2. B-cells and antigen presentation

CD82 is also expressed on CD19+ B-cells [15]. Coimmunoprecipitation studies revealed that CD82 was present in complexes with MHCII receptors, B-cell costimulatory molecules CD19 and CD21, tetraspanins CD9, CD53, CD63, and CD81, and several integrins—α4β1, α6β1, and α5β1 [70–73]. Recent studies in CD81 null mice demonstrate that CD81 is essential for assembly and localization of the CD19 costimulatory complex in B-cells [74]. While CD9, CD53, and CD82 also form complexes with CD19/ CD21, their role in B-cell activation is still unknown. T-cell and B-cell activation is mediated by interactions with MHC molecules on antigen presenting cells (APCs). CD82 is also found in association with peptide-loaded HLA-DR MHC II complexes on intracellular membrane compartments in APCs. CD82 appears to facilitate transport and clustering of the loaded MHC II complexes on the cell surface [75].

2.2.3. Animal models

To date six tetraspanins have been deleted from the mouse genome. Each tetraspanin appears to have a unique biological role as well as overlapping roles with other tetraspanins (Table 1). For instance, only loss of CD9, and to a lesser extent loss of CD81, leads to a fertility defect due to impaired egg-sperm fusion [76–79]. CD81, but not CD9 or TSSC6, plays a critical role in B-cell activation by regulating expression of the BCR costimulatory molecule CD19 [80,81]. Both CD9 and CD81 are important in neurons, but in different cell types [82–84]. CD37, which is exclusively expressed in immune cells, is required for efficient signaling in both T- and B-cells [85,86] and penumbra (Tspan33) is required for efficient erythopoiesis [87]. Loss of either CD151 or TSSC6 leads to defects in platelets, while loss of CD151, but not TSSC6, also disrupts kidney function, impairs pathological angiogenesis, and inhibits wound healing [88–93]. Genetic deletion of CD82 has not yet been formally reported, but preliminary studies from our lab indicate that CD82 null mice are viable and fertile (Miranti, unpublished data). Phenotypes associated with loss of two or more tetraspanins await further analysis.

2.3. CD82 as a metastasis suppressor

Based on studies in immune cells, CD82 is important for cell signaling, cell adhesion, and sorting/trafficking of proteins to the cell

surface. Are these same functions relevant to its role in metastasis suppression, or are there additional functions for CD82 in non-immune cells? It is important to point out that although CD82 loss is associated with metastasis, up-regulation of other tetraspanins, namely CD151 and CO-029 is associated with more aggressive disease [94,95]. Is CD82 association with other tetraspanins critical for its suppressive function and does CD82 act to inhibit their activity? What are the metastasis "accelerators" that CD82 suppresses? Finally, are there functional links between loss of CD82 and loss of the other metastasis suppressor genes, and how many metastasis suppressor functions need to be removed to generate a metastatic cancer cell? All these questions remain largely unanswered, but progress is being made in all areas.

2.4. Cell-matrix adhesion

The most well characterized CD82 function in non-immune cells is its role in integrin-mediated cell migration on extracellular matrix. The logical connection to metastasis suppression is that enhanced integrin-mediated migration is crucial for detachment of tumor cells from their local microenvironment, followed by migration and penetration into the blood and lymph system. Consistent with this are numerous reports demonstrating that re-expression of CD82 in metastatic tumor cells inhibits in vitro migration and matrigel invasion [3,114,95a,5,95b,98,99]. Additionally, the integrin/CD82 associations discovered in immune cells were found to exist in non-immune cells. Immunofluorescent staining of MDA-MB-231 breast cancer cells adherent to a laminin matrix demonstrated colocalization of CD82 with several tetraspanins, CD9, CD63, CD81, and CD151, within α3β1-containing adhesion structures. CD82 was present in α3β1 immunoprecipitates, along with CD81 and CD151. Talin and MARCKS, known PKC substrates, were present in the adhesion structures containing the tetraspanins, but FAK and vinculin were not, consistent with a role for tetraspanins in regulating early adhesion/migration events [96]. CD82 has subsequently been reported to coimmunoprecipitate with α3β1, α6β1, and α5β1 in various adherent cells (Table 2) [29,49,97].

Reduced adhesion-induced signaling has been observed in CD82-re-expressing metastatic tumor cells. Changes in integrin-mediated signaling include reduced Src activation and phosphorylation of its downstream targets, i.e. p130Cas and Src-dependent FAK phosphorylation sites, as well as alterations in p130-Cas/CrkII complex formation [98,99]. Direct interference with these pathways reduced cell migration and invasion, as did CD82 re-expression. CD82 was shown to impact Rho GTPase signaling in immune cells; however, this has not been directly investigated in the CD82 re-expressing tumor cells [66]. Nonetheless, reduced signaling via p130Cas/CrkII would be predicted to inhibit Rac signaling. Strikingly, CD82 appears to have little or no effect on integrin-mediated activation of the Ras/Erk or PI-3K/Akt signaling pathways, suggesting specificity with respect to the types of signaling pathways that CD82 impacts (Table 2).

One study demonstrated that PMA treatment of immune cells induced PKC association with CD82 as well as CD9, CD53, CD81, and CD151. CD81 and CD151 could be cross-linked to PKC indicating a direct association. Whether this was also true for CD82 was not mentioned [100]. PMA treatment also induced an association between PKCα and the extracellular domain of α3β1 integrin. It was subsequently shown that PKC can bind to the β1 integrin tail and regulate cell motility [101,102]. No follow-up studies in metastatic tumor cell lines re-expressing CD82 have been reported. However, the association between CD82 and the PKC substrates talin and MARCKS in adhesion structures [96] suggests there is likely to be an association, even if it may be indirect (Table 2). Furthermore, the role of PKC in regulating actin dynamics in cell migration is well documented [103]. PKC-mediated phosphorylation of several substrates, MARCKS, adducin, fascin, talin, and ERMs, reorganizes existing actin structures allowing for the reassembly of new actin structures that promote migration. It is possible that CD82 suppresses cell motility through a negative influence on PKC (Fig. 1).

In contrast to CD82, CD151 enhances and is required for cell migration. CD151 not only associates with PKC [100], but also associates with PI-4K [104]. $PI(4,5)P_2$ interacts with MARCKS and is involved in recruiting actin remodeling proteins upon dissociation of MARCKS following PKC-mediated phosphorylation [103]. Thus, in the absence of CD82, CD151 could drive cell migration by coordinating PKC and PI-4K signaling to drive actin reorganization. When CD82 is present it may sequester PKC away from CD151 and limit its ability to stimulate cell migration.

Another model for CD82-mediated inhibition of cell motility is provided by studies on tetraspanin-associated proteins EWI-2 and EWI-F. The single-pass Ig superfamily membrane proteins EWI-2 and EWI-F were originally identified as CD9- and CD81-associated proteins in chemical cross-linking studies [105–108]. Subsequently, EWI-2 was found in close association with CD82 in a complex with CD81 [109](Table 2). Over expression of EWI-2 in metastatic prostate cancer cells inhibited cell migration and further synergized with CD82 [109]. Over expression of EWI-2 in A431 cells, which do not have CD82 [110], also suppressed their migration [111]. EWI-2 enhanced α3β1/CD81 complex formation in A431 cells, suggesting that the α3β1/CD81complex is inhibitory to migration. The EWI-2 cytoplasmic domain was required for its inhibitory function. Pulldown experiments demonstrated a direct interaction between ERMs and the cytoplasmic domain of EWIs. The exact amino acids involved in this interaction were not determined, but the highly charged basic residues maybe involved. A dominant negative form of moesin delocalized EWI. Inhibition of EWI-2 expression by siRNA stimulated cell migration and increased phosphorylation of ERMs [112]. Thus CD82 may simultaneously function to inhibit PKC activity, while also controlling localization of EWI-2 protein, ultimately limiting actin remodeling events and migration by controlling ERMs (Fig. 1).

2.4.1. Integrin trafficking

PKCα activation stimulates β1-dependent migration and induces β1 integrin internalization and endocytosis. PKCα stimulated migration is inhibited by blockade of endocytosis [101]. Several tetraspanins, including CD82, contain an endosomal sorting motif (Tyr-X-X-φ) in the C-terminal tail and tetraspanins are often found on internalized vesicles [51,113]. It is possible that tetraspanins influence PKC-mediated internalization of integrins. The reduced cell adhesion observed in some CD82 re-expressing cells could be due to reduced cell surface integrin expression due to changes in integrin internalization [6,29,114,115]. For instance in one study, CD82 re-expression in a metastatic prostate cancer cell line reduced α6 integrin surface levels, reduced cell adhesion, and enhanced α6 internalization [97]. This result is somewhat unexpected since increased internalization would

Table 2
CD82-associated and effector proteins

Extracellular
DARC/duffy antigen
Gangliosides: GD1a, GM2, GM3
Membrane associated
Tetraspanins: CD9, CD81, CD151, CD63
Integrins: α3β1, α6β1, α5β1, α4β1
Type I TM: EWI-2, GGT, MHC II
Receptors: EGFR, c-Met, TCR
Type III TM: VangL1
Intracellular
PKC
Src
Rho GTPases

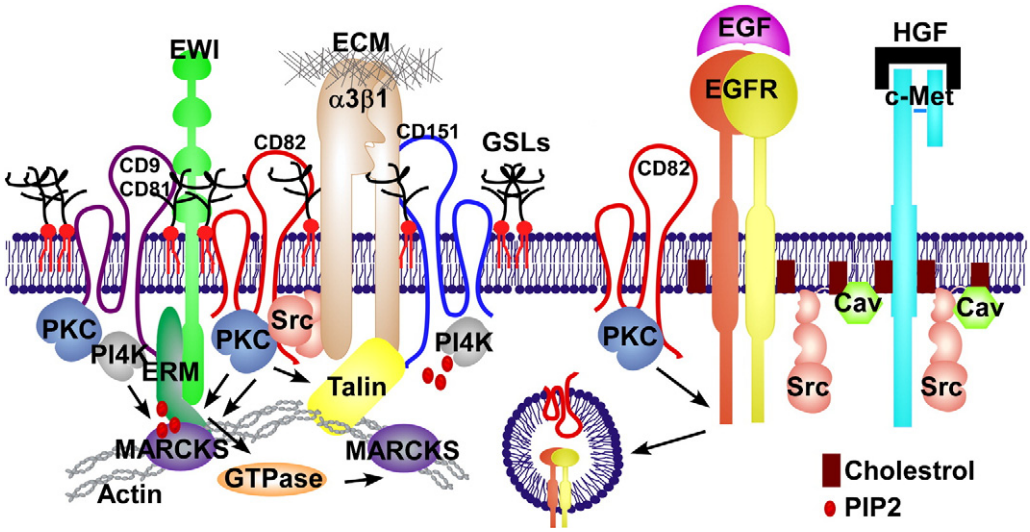

Fig. 1. Model for CD82 regulation of integrin and receptor tyrosine kinase signaling. Integrin Signaling: CD82 in association with other tetraspanins (CD9, CD81, CD151) within the ganglioside (GSLs) enriched microdomains control the signaling output from integrins (α3β1) and associated proteins (EWI) to tightly control early cytoskeletal rearrangements associated with integrin engagement with the matrix (ECM). Talin association with integrin tails and ERM association with EWI are both targets of PKC signaling, which affects their ability to bind actin and control actin polymerization. The actin binding protein MARCKS is targeted by PKC as well as Rho GTPases. Src also controls actin dynamics. In addition, actin polymerization requires the production of PIP2 (red spots) via PI-4K, which binds both ERM and MARCKS. It is not know whether CD82 association with PKC enhances or inhibits its activity within the complex, but CD82 has been shown to inhibit Src. RTK Signaling: The ability of CD82 to inhibit EGFR signaling by its ligand EGF within caveolin (Cav) cholesterol-rich lipid rafts (brown bars) is mediated by PKC-dependent phosphorylation of EGFR cytoplasmic tail and its subsequent internalization. Whether the same mechanism is true for regulating HGF-induced c-Met activity is unknown.

be expected to increase cell migration, when in fact CD82 suppresses migration in these cells [98]. However, it was not clear whether the internalization studies were done with cells plated on the relevant matrix. The total cellular levels of α6 integrin were unchanged, indicating that CD82-mediated internalization does not lead to degradation of the integrin. The effect of CD82 on internalization of other integrins was not monitored.

The cell surface expression of α3 or α6 integrins was not altered in CD151 null cells generated from mutant mice [90] or by siRNA-knock down [116,117]. However, the internalization rate of α3β1 in these cells was reduced, which is consistent with reduced migration due to reduced integrin turnover. Mutation of the sorting motif in the C-terminal cytoplasmic domain markedly attenuated CD151 internalization and blocked migration; however, only subtle changes were observed in integrin internalization [118]. It is possible that the ability of the CD151/α3β1 complex to associate with other tetraspanins, which also have internalization motifs, may be responsible for facilitating efficient α3β1 internalization. Alternatively, inhibition of cell migration involves additional changes. A direct effect of the CD82 sorting motif on integrin internalization has not been reported.

Does CD82 influence integrin synthesis and/or presentation at the cell surface? Other tetraspanins have been shown to regulate early biosynthesis of their associated molecules. Examples include CD151/α3β1 [119,120], uroplakins/UPs [121], CD81/CD19 [122], and CD9/pre-β1 integrin [123]. In one report, re-expression of CD82 in tumor cells negatively influenced pre-β1 integrin processing resulting in reduced β1 integrin on the cell surface [124]. Tetraspanins may influence integrin trafficking; however, currently the link to CD82-mediated suppression of metastasis is weak.

2.4.2. Integrins vs tetraspanins

Despite extensive evidence that CD82 inhibits integrin-dependent signaling and migration, the evidence that CD82 suppresses metastasis via this mechanism is lacking. An analysis of the domains within CD82 that are responsible for 1) interaction with integrins, 2) suppressing integrin-mediated migration/invasion, and 3) metastasis suppression is required. While CD82 can colocalize and be coimmunoprecipitated with several different integrins, this interaction appears to be indirect since it is only observed in mild detergents.

A direct interaction between the α3 and α6 integrins and CD151 was demonstrated by their ability to be chemically cross-linked, stability of the association in harsh detergents, and identification of the interacting domains by mutagenesis [119,120,125]. CD151 interacts with the extracellular stalk domain of α3 (aa 570–705), a region not directly involved in mediating cell adhesion [125]. The integrin-binding region of CD151 mapped to an 11 amino acid region within the EC2 domain (aa 195–205) containing the sequence QRD (aa 194–196) [119,120]. Mutation of the α3 binding region in CD151 had no affect on the ability of CD151 to associate with itself or other tetraspanins (CD9, CD81) [120]. However, CD151 containing a QRD mutation disrupted α3/α6-dependent formation of cellular cables on Matrigel and altered cell spreading [119]. In fact, removal of CD151 from cells disrupts α3/α6 association with other tetraspanins and impairs integrin internalization [117,126]. Thus any effects CD82 may have on α3 or α6 integrin-mediated events are likely to involve interactions with CD151.

CD82, like all tetraspanins thus far investigated, is palmitoylated [50,127–130]. Palmitoylation at membrane proximal cysteines promotes protein–protein interactions between weakly associated proteins and is important for lipid raft association of signaling molecules [131]. In fact, inhibition of tetraspanin palmitoylation significantly reduces tetraspanin–tetraspanin interactions [129,132]. Mutation of all five membrane proximal cysteines in CD82 abolished palmitoylation, reduced but did not abolish association with CD9 and CD81, and failed to suppress migration and invasion of tumor cells. The CD82 palmitoylation mutant also no longer suppressed p130Cas-CrkII signaling [50]. However, the effect of palmitoylation loss on association with integrins or metastasis was not reported.

The integrins that specifically associate with tetraspanins, namely α3, α6 and β4, are also palmitoylated. Mutation of the 7 potential palmitoylation sites in β4 integrin impaired cell spreading and reduced signaling to p130Cas. β4 association with tetraspanin complexes was reduced upon loss of β4 palmitoylation [133]. Although the ability of CD82 to suppress integrin-mediated events is dependent upon its palmitoylation, it is not clear whether it is the association with other tetraspanins or integrins or both that is important.

Palmitoylation promotes the association of proteins with lipid rafts. However, the evidence that tetraspanins associate with classical

cholesterol-rich lipid rafts is limited. Instead, tetraspanins are most commonly associated with ganglioside-enriched membrane microdomains (GEM or TEM). This observation has been made primarily in "resting" cells, i.e. cells in which specific signaling pathways have not been stimulated. However, in immune cells, co-crosslinking of BCR and CD19 causes BCR, CD19, and CD81 to appear in cholesterol-rich lipid rafts, which is required for Vav and PLCγ recruitment into the rafts [134]. Furthermore, the level of palmitoylation was increased specifically in lipid raft-associated CD81. Blockade of palmitoylation prevented CD19/CD81 association with rafts. Thus, under some stimulatory conditions there may be significant shifting of tetraspanins from GEM/TEMs into rafts, especially during active signaling processes. These findings also support the idea that palmitoylation of tetraspanins may be regulated, as has been observed for other molecules [131].

A class of 23 enzymes named for the Asp–His–His–Cys active site motif (DHHC), are involved in the transfer of palmitoylate to target cysteine residues. DHHC2, 5, 7 and 11 were identified in tetraspanin complexes by mass spec. Of these, DHHC2 was most efficient at stimulating palmitoylation of CD9 and CD151. DHHC2-dependent palmitoylation promoted interactions between CD9 and CD151, but had no affect on integrin β4 palmitoylation or association of α3 integrin with CD151. Mutation of the active DHHC motif blocked tetraspanin palmitoylation. Furthermore, loss of DHHC2, but not 6 other DHHC proteins, not only diminished CD9 and CD151 palmitoylation, but also dramatically enhanced their degradation. Loss of DHHC2 reduced cell–cell contact, probably as a result of general loss of tetraspanins due to degradation [135].

Could tetraspanin interactions influence the function of each other? CD82 appears to regulate cell functions that other tetraspanins enhance. For instance, CD151 and CO-029 have the opposite effects on cell migration as CD82 and both are over expressed in cancers. Blocking CD151 function with antibodies or inhibiting expression with siRNA preduces cell migration and invasion, suppresses signaling to FAK, Rac1, and Src kinases, and delays tumor progression in mice [117,126,136]. CO-029 enhances cell migration through its association with α6β4 and CD151 in invasive cancer cells [136a,136b]. CD9 generally has the similar suppressive effects as CD82, but this is dependent on cell type. Recently Net-6 (Tspan13) was demonstrated to behave as a tumor suppressor [137]. The apparently opposing actions of different tetraspanins, suggests that CD82 re-expression in tumor cells may restore the tetraspanin "balance" to specifically impose tighter regulatory controls on integrin-mediated motility. The converse of that, i.e. loss of CD82, would cause an "imbalance" in tetraspanins that favors enhanced motility. More rigorous experimentation is required to sort this out.

2.5. Growth factor receptor signaling

Are integrins the only molecules that CD82 regulate? Tetraspanins play a critical role in regulating receptor tyrosine kinase signaling in immune cells, i.e. TCR and BCR [66,134]. In addition, signaling through the receptor tyrosine kinase c-Kit is inhibited when it associates with tetraspanins in myeloid cells [138]. CD82 has been shown to affect signaling mediated by at least 2 receptor kinases present in non-immune cells, EGFR and c-Met.

2.5.1. EGFR and c-Met

In a normal mammary epithelial cell line, HB2, CD82 expression reduced EGF-stimulated wound migration, EGFR and Shc tyrosine phosphorylation, and Grb2 association with EGFR [132]. CD82 expression increased EGF-induced EGFR internalization. CD9 was shown to have a similar effect on EGFR in several tumor cell lines [139]. EGFR complexes containing CD9 or CD82 could be detected, but integrins were not present in these complexes.

Re-expression of CD82 in metastatic prostate cancer cell lines reduced both ligand-dependent and -independent activation of the HGF/SF receptor c-Met, and suppressed c-Met-dependent invasion [99]. c-Met signaling and invasion could be rescued at high concentrations of HGF, suggesting that CD82 modulates the level of c-Met activation rather than completely suppressing it. c-Met could not be detected in CD82 complexes, nor did it significantly colocalize with CD82 in cells, suggesting an indirect mechanism of regulation. Similarly, HGF-induced cell migration as well as Grb2 and p85 association with c-Met was reduced upon CD82 re-expression in H1229 lung carcinoma cells [140]. In contrast, removal of CD151 by siRNA in salivary gland adenoid cystic carcinoma tumor cells severely reduced HGF-stimulated cell migration, while CD151 over expression increased c-Met-mediated migration [141].

2.5.2. Gangliosides

Glycosphingolipids (GSLs) are associated with organized membrane microdomains. Gangliosides in particular modulate 1) growth factor-stimulated receptor tyrosine kinases, 2) integrins complexed with tetraspanins, and 3) downstream signaling molecules such as Src and small G proteins [142]. The ability of tetraspanins, and in particular CD82, to modulate RTK and integrin signaling may be mediated by gangliosides, which are found within the tetraspanin-enriched microdomains [143].

The ability of CD9 to promote tumor cell motility is suppressed by the exogenous addition of GM3 ganglioside. This is specific, in that addition of GM1 did not have a similar effect. IdID mutant CHO cells that are defective in UDP-Gal 4-epimerase, cannot synthesize gangliosides unless cultured in the presence of galactose. Expression of CD9 promotes motility in the absence of galactose, which is suppressed when galactose is present [144]. CD9/α3 integrin complexes could be detected by co-immunoprecipitation only when GM3 was present [145]. A similar relationship was observed in human bladder cancer cell lines; cells with higher GM3 levels are much less invasive than those with lower levels. A stronger interaction between α3 integrin and CD9 was detected in high GM3 expressing cells. The non-invasive cells could be converted to invasive cells by depletion of GM3 or siRNA knockdown of CD9 and vice versa. GM3 addition induced Csk translocation into GEMs and inhibited Src activation [146]. Interestingly, v-Jun-induced cell transformation greatly reduces the levels of GM3 and GM3 synthase mRNA in fibroblast cell cultures. Re-expression of GM3 synthase suppressed growth in agar and increased the levels of CD9/α5β1 complexes in those cells [147]. Thus suppression of migration is mediated by enhanced complex formation between GM3/CD9/integrins, suggesting this complex limits migration by diminishing integrin function.

The level of GD1a ganglioside increased and colocalized with CD82 on the cell surface when CD82 was re-expressed in normal mammary cells [148]. Specific removal of GD1a decreased CD82 association with tetraspanin CD151, but increased CD82 association with EGFR [149]. Unfortunately, the effect of specifically depleting GD1a on EGFR signaling was not determined. In a separate study CD82-induced suppression of EGFR phosphorylation and internalization was shown to be dependent on an association between PKCα and CD82 and the lipid raft protein caveolin-1. In this model EGF-induced EGFR internalization is regulated by PKCα-mediated phosphorylation of EGFR at Thr654. The ability of the caveolin-1/CD82/PKCα complex to inhibit EGFR signaling was dependent on GM3 [150]. Thus "mixing" between membrane microdomains, i.e. caveolin-containing lipid rafts and GEM/TEMs, may occur subsequent to activated signaling and function to limit the signaling response (Fig. 1).

GSLs impact c-Met signaling as well. Re-expression of CD82 in an invasive bladder cancer cell line reduced c-Met signaling in response to low doses of HGF and inhibited HGF-dependent motility. Removal of GM2 prevented CD82-mediated suppression of c-Met activation and motility. GM2, but GM3, could be detected in a complex with CD82 [151]. The level of ganglioside expression clearly has an impact on the ability of tetraspanins to regulate RTK and integrin signaling.

Together the integrin and growth factor receptor studies suggest that CD82 functions to suppress cell migration through the dynamic assembly and disassembly of carbohydrate-enriched complexes consisting of tetraspanins and integrins within GEMs/TEMs. These interactions are critical for regulating signal transduction pathways that impact cell migration via integrins. In addition, CD82 can negatively impact RTK signaling within in lipid rafts by limiting signaling and increasing internalization. There is specificity in that CD82 exerts its suppressive effects on migration and RTKs primarily by targeting Rho, PKC, and Src signaling pathways and has a minimal impact on proliferation-regulated pathways (Fig. 1). This fits well with the known affects of CD82 loss on tumors in vivo.

2.5.3. GPCR

Secreted tissue transglutaminase (TG2) is a Ca^{2+}-activated extracellular matrix protein involved in matrix cross-linking, which binds fibronectin and integrins, and stimulates cell adhesion, spreading, and focal contact assembly [152]. TG2 levels are decreased in aggressive tumors and metastases [153]. Expression of GPR56, recently identified as the TG2 receptor [154], is dramatically reduced in highly metastatic cells [154,155]. Over expression of GPR56 suppresses tumor growth and metastasis, while reduced expression enhances tumorigenesis and metastasis.

GRP56 has homology with the LNB-TM7 subfamily of GPCRs. The N-terminus of these molecules contains adhesion molecule domains. The planar cell polarity cadherin-like protein, Flamingo (Celsr1), belongs to this class of GPCRs [156]. GPR56 is highly expressed in glial cells, particularly in regions that contact the basement membrane. Genetic mutations in GPR56 cause congenital brain malformation in humans, and GPR56 is required to maintain basement membrane integrity during cortical development [157].

GPR56 was also identified as a CD81-associated protein in retinoic acid-differentiated NT2 teratocarcinoma cells by mass spectrometry. GRP56 and the heterotrimeric G protein subunits, Gα(q/11) and Gβ, associated with both CD9 and CD81, but not CD151 or CD63. CD81 antibody caused dissociation of Gα(q/11) from GPR56/CD81, while PMA treatment induced GPR56 dissociation and internalization away from the CD81/ Gα(q/11) complex [158]. Thus, depending on the stimulatory signal, CD81/CD9 may differentially regulate signaling through GPR56. The link between CD81 and GPR56 is interesting given the dramatic overproduction of glial cells during brain development in CD81-null mice. The defect in CD81 null and GPR56 mutant glial cells may be linked to the role of tetraspanins in trafficking. Human genetic mutations in GPR56 that map to the N-terminal domain result in poor surface expression due to reduced intracellular trafficking [159]. Reduced GPR56 surface expression would be expected to increase the proliferation of glial cells.

CD82 is not well expressed in the brain, but is present in many other tissues that develop tumors. Links between CD82 and GPR56 or other GPCRs have not been reported.

2.6. Invasion

Thus far, we have focused primarily on adhesion and migratory events regulated by integrins and tetraspanins. However, metastasis requires the activation of extracellular proteases that degrade the extracellular matrix to facilitate cell invasion and movement away from the primary tumor. Several major protease systems are known to be activated in invasive cancer cells and three have been linked to tetraspanins; a disintegrin and metalloprotease (ADAMS), urokinase plasminogen activator and receptor (uPA/uPAR), and matrix metalloproteases (MMPs).

2.6.1. ADAMS

ADAMs are transmembrane proteases whose activity is restricted to membrane localized substrates. One major function of ADAMs is the shedding of cell surface growth factor ligands such as HB-EGF and TGFα leading to paracrine and autocrine activation. The disintegrin and cysteine-rich domains on the extracellular domain of ADAMS also allows for interactions with integrins or syndecans to facilitate cleavage of the ECM [160,161]. CD9 is known to associate with HB-EGF and TGF-α and regulate their juxtacrine signaling activity [162,163]. GPCR signaling stimulated the association of HB-EGF with CD9 and enhanced cleavage of HB-EGF, which was dependent on ADAM10 [164]. Other studies suggest that ADAM17 is the primary enzyme involved HB-EGF and TGF-α shedding [165]. Association of ADAM17 with tetraspanins has not been reported. Fertilization studies indicate critical roles for CD9/CD81 and ADAM2 in egg-sperm interactions, such that loss of these molecules on eggs and sperm respectively inhibit fertilization [76,77,79,166,167]. However, the exact functional relationship between ADAMs and tetraspanins in sperm–egg fusion remain controversial. Despite demonstrated interactions between tetraspanins and ADAMs, the effect of tetraspanins on ADAM activity has not been determined. ADAMs are known to be involved in matrix degradation in metastatic lesions, but any links to tetraspanins in this context have not been reported.

2.6.2. uPA and uPAR

Binding of urokinase-type plasminogen activator (uPA) to its receptor (uPAR) locally cleaves plasminogen leading to generation of the diffusible protease plasmin. uPAR has been shown to associate with several integrins including α3β1 and α5β1. uPAR binds directly to the beta propeller of β1 integrins and affects many functional aspects of integrins during migration [168,169]. Thus given the close association between uPAR and integrins, and integrins and tetraspanins, it is highly likely that uPAR activity may be regulated by tetraspanins. CD82 expression in a normal mammary epithelial cell line, HB2, was shown to significantly reduce plasminogen activation. Reduced plasminogen activation was due to reduced uPA binding to uPAR in the presence of CD82, but was not mediated by an interaction between uPAR and CD82. Instead, CD82 expression led to a redistribution of uPAR to α5β1 containing focal adhesions [170].

The role of CD82 in regulating uPAR activity in a metastatic tumor setting has not been investigated. However, it has recently been shown that uPAR activation in metastatic prostate cancer cells generates a cleaved form of α6 integrin that promotes increased migration and invasion on laminin substrates [171]. Furthermore, c-Met activation via HGF has been shown to stimulate uPAR activity in MDCK cells and in metastatic prostate cancer cells [172,173]. If CD82 negatively regulates both c-Met and uPAR activity, then CD82 should suppress generation of the cleaved α6 integrin in metastatic cells.

2.6.3. MMPs

A role for MMPs in tumor angiogenesis, tumor invasion, and establishment of metastases at secondary sites has been well established [174]. Antibody-mediated ligation of α3β1-tetraspanin (CD9, CD63, CD151, CD81) complexes in MDA-MB-231 cells stimulated the production of MMP-2, but not MMP-9, and increased invasiveness [175]. Elevated CD9 expression in a melanoma cell line induced MMP-2, but suppressed MMP-9 expression [176]. Whereas expression of CD151 in the same cell line stimulated MMP-9 expression [177]. Elevated MMP-2 and MMP-9 expression was dependent on cell adhesion to laminin, signaling through Jnk, and lead to increased AP-1 binding on the two promoters. Re-expression of CD82 in H1299 lung carcinoma cells reduced MMP-9 activity, but elevated expression of the metalloprotease inhibitor TIMP1 [178].

Clearly there are functional relationships between tetraspanins and extracellular proteases. However, the mechanisms involved in tetraspanin-dependent regulation of their activity and the overall importance in metastasis suppression or promotion still needs to be determined.

2.7. Homotypic cell–cell interactions

An under-appreciated, but highly relevant, aspect of CD82 function is its role in cell–cell adhesion. Numerous studies have demonstrated that CD82 is localized at and induces cell–cell adhesion [178a,68,178b,115,114,95a]. The exact nature of the cell–cell interaction has not been deciphered, except the interaction is in some cases Ca^{+2}-independent [178b,114,95a]. Loss of the cell–cell adhesion molecule E-cadherin is highly associated with metastasis in epithelial cancers and is considered to be a metastasis suppressor. However, the Ca^{+2}-independence of CD82-mediated adhesion rules out E-cadherin as a direct target, suggesting that in addition to E-cadherin loss, metastasis may require disruption of additional cell–cell adhesion molecules that are regulated by CD82.

2.7.1. Claudins

One type of Ca^{+2}-independent cell adhesion molecule that could be regulated by CD82 is claudins, which form tight junctions in polarized cells. A few family members are known metastasis suppressors. Tumorigenic changes in cellular localization and expression of claudins have been reported [179], although exactly which claudin is changed in which cancer type remains controversial. While CD82 itself has not been directly implicated, several examples of direct associations between tetraspanins and claudins have been reported.

In a yeast two hybrid screen, the C-terminal half of tetraspanin OAP1/Tspan3 was found to interact with claudin11; which forms tight junctions in myelin sheaths of the CNS. OAP1, claudin-11, and β1 integrin formed a complex in oligodendrocytes. Claudin-11/OAP1 interactions were involved in promoting proliferation and cell migration of oligoendrocytes in vitro [180]. Claudin-7, EpCAM (another adhesion molecule), tetraspanin CO-029, and CD44v6 form a complex in gastrointestinal tumors. Co-expression of these 4 markers is inversely correlated with disease-free survival in human colon cancer. Claudin-7 was required for EpCAM to associate with CO-029 and CD44v6 [181]. Claudin-1 was shown to directly interact with CD9 based on chemical cross-linking studies in non-polarized A431 cells. Claudin-1 also interacted with CD81 and CD151. Interestingly in polarized epithelial cells, CD9/claudin 1 complexes were not detected [182]. It should be noted that the claudin studies involving tumor cells primarily focused on tetraspanins known to potentiate tumor aggressiveness, i.e. CO-029, CD9, CD151, and suggest that these tetraspanin associations with claudins in tumor cells may act to inhibit tight junction formation. Most of these tumor cells do not express CD82, and most normal polarized epithelial cells do. One possibility is that re-expression of CD82 could shift tetraspanin/claudin interactions to restore cell–cell adhesion (Fig. 2A).

2.7.2. E-cadherin

CD82 re-expression in h1299 non-small cell lung carcinoma cells increased homotypic cell–cell aggregation, which was dependent on calcium and blocked by anti-E-cadherin antibody. Surprisingly, CD82 expression did not alter E-cadherin distribution, which was present at the cell surface at cell–cell junctions. However, β-catenin distribution was altered [183]. Over expression of CD151 in A431 cells also enhanced E-cadherin-based cell–cell adhesion. cdc42 and Rac were elevated in the CD151-overexpressing cells and CD151-induced cell–cell adhesion was dependent on PKC [184]. Conversely, immortalized epithelial cells taken from mice lacking α3 integrin or treated with CD151 siRNA displayed reduced cell–cell adhesion. Loss of α-actinin association with E-cadherin, increased β-catenin tyrosine phosphorylation, and increased stress fibers (indicative of elevated Rho activity) were also seen in α3 null and CD151 siRNA cells [185]. However, E-cadherin along with β-catenin remained associated with the cell membrane. Thus tetraspanins can regulate E-cadherin dependent adhesion, not by regulating E-cadherin directly, but by regulating the association of the E-cadherin/β-catenin complex with the cytoskeleton to stabilize cell–cell adhesion. Surprisingly, CD151 and CD82 behave the same way, i.e. both increase cell–cell adhesion. However, the consequences of increased cell–cell adhesion may be different as a result of specific changes in downstream signaling pathways.

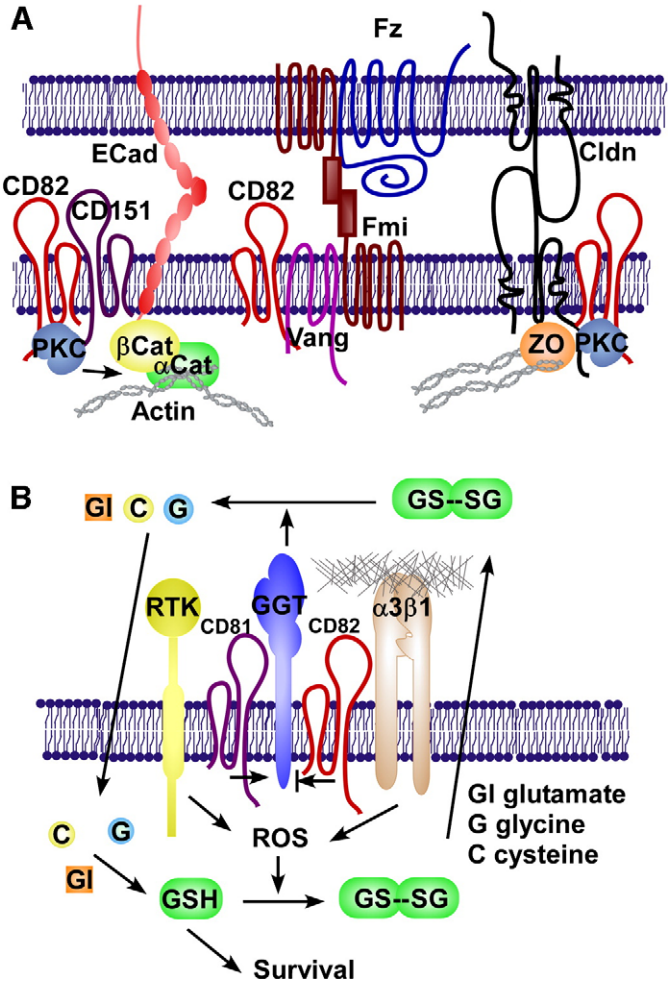

Fig. 2. A) Model for CD82 regulation of homotypic cell–cell interactions. The ability of CD82 and other tetraspanins (CD151) to affect adherins junction E-cadherin (ECad) mediated cell–cell interactions is regulated at the level α-catenin (αCat) and β-catenin (βCat) interactions with the actin cytoskeleton. PKC is involved in regulating those interactions. CD82 can also directly interact with VangL1 (Vang), which is involved in establishing cell polarity through interactions with the atypical cadherin Flamingo (Fmi) in one cell and Flamingo and the Wnt receptor Frizzled (Fz) in the adjoining cell. The effect of CD82 on this interaction is unknown. Tetraspanins can also associate with claudins (Cldn), which are normally linked to the actin cytoskeleton via ZO proteins at tight junctions. PKC signaling regulates ZO activity. Whether CD82 controls PKC signaling at tight junctions has not been determined. B) Model for CD82 regulation of oxidative stress. CD81 and CD82 both associate with the transmembrane γ-glutamyl transpeptidase (GGT), which catalyzes the first step in the degradation of GSH and GSH-conjugates (GS–SG) into glutamate (Gl), cysteine (C) and glycine (G). These are transported back into the cell to regenerate GSH. GSH is responsible for reducing ROS generated by local signaling through RTKs or integrins (α3β1). The correct balance ensures cell survival. The effect of specific tetraspanins on GGT activity is unknown.

The downstream signaling pathways required for CD82-mediated E-cadherin adhesion have not been investigated. However, the molecules involved in CD151-induced cell adhesion have been. CD151 and α3β1 integrin were both required for efficient expression of PTPμ, a transmembrane protein tyrosine phosphatase involved in cadherin-mediated adhesion. In addition, E-cadherin and β-catenin could be detected in an α3β1/CD151 complex containing PTPμ, PKCβII, and RACK1. Loss of α3 disrupted these interactions. The stalk domain of α3 integrin, required for interaction with CD151, was sufficient to

rescue PTPμ expression, restored the multi-complex, and increased cell–cell adhesion in α3 null cells. These data suggest a strong requirement for α3β1 in cell–cell adhesion. In fact, it was demonstrated that α3β1 within the cadherin–catenin complex was distinct from that involved in adhesion to laminin. What will be most valuable is to determine how CD82 impacts these interactions (Fig. 2A).

2.7.3. PCP and Wnt

Vangl1 was pulled out of a two hybrid screen using a C-terminal fragment of CD82. Loss of Vangl1 in CT-26 colon cancer cells reduced, while over expression enhanced, adhesion and invasion. Vangl1 over expression did not increase tumorigenesis or metastasis, but loss of Vangl1 decreased growth and inhibited metastasis. Vangl1 levels were higher in human gastric tumors and metastases, compared to normal tissues [186]. These data suggest that Vangl1 promotes a more metastatic phenotype. Over expression of Vangl1 reversed the suppressive effects of CD82 on matrigel invasion, suggesting that Vangl1 is downstream of CD82.

The most well characterized role for Vang-like proteins (aka strabismus) are their involvement in establishing planar cell polarity (PCP) in epithelia during eye and wing development in Drosophila [187]. Strabismus functions in the context of Frizzled (a Wnt receptor) to communicate cell polarity. Flamingo, an atypical cadherin of the LNB-TM7 subfamily of GPCRs, functions to establish an asymmetric cell–cell interaction by causing recruitment of a Frizzled complex containing Disheveled and Diego at the cell–cell interface in one cell and a Strabismus/Prickle complex at the same cell–cell interface in the other cell [187,188]. Exactly how Flamingo establishes the asymmetry is not clear, but may involve two functional forms of Flamingo on either side of the membrane (Fig. 2A).

Mammals have two strabismus-like proteins, Vangl1 and Vangl2, as well as mammalian homologs of the other PCP proteins. Mice lacking Vangl1 are viable and fertile, but do display subtle alterations in polarity of inner hair cells of the cochlea, a PCP-dependent process. The Vangl2 null mice are embryonic lethal with defects in neural tube closure. Double heterozygotes have developmental defects in the neural tube, inner ear, and heart [189]. The mild phenotype in Vangl1 null mice suggests Vangl1 may have cell–cell functions independent of PCP. Thus Vangl1 is well poised to function as a CD82-regulated target to control cell–cell interactions.

Intestinal trefoil factor (ITF) is a secreted factor that promotes the migration of intestinal epithelial cells during mucosal repair. Vangl1 is Ser/Thr phosphorylated in response to ITF and removed from the cell membrane following ITF stimulation. Loss of Vangl1 inhibited ITF-dependent migration [190]. This function of Vangl1 is consistent with its role as a pro-metastatic factor; however, the HT29 cells in which Vangl1 function was assessed, express CD82 [191]. The internalization and removal of Vangl1 from the membrane would be consistent with a role for CD82 in regulating trafficking of Vangl1. The exact relationship between CD82 and Vangl1 needs to be better characterized.

An extracellular p90K Vangl1-interacting protein was detected in a two hybrid screen [192]. p90K is a soluble oligiomerized ligand for galectin receptors. It is a strong inducer of ICAM-1 and VCAM-1 expression on tumor endothelium [193]. Both ligand and receptor are strongly up-regulated in tumors and associated with metastasis and poor prognosis. Interestingly, p90K can mediate homotypic cell–cell adhesion via galectins on adjacent tumor cells and can enhance cell matrix adhesion. These processes may be critical for cancer cell survival in the bloodstream and during the establishment of metastatic colonies [194]. Thus p90K/galectin has the potential to play a role in many steps in tumor progression and metastasis. However, the connection between p90K, Vangl1, and CD82 remain puzzling. If Vangl1 acts to promote metastasis [186], then it would be expected to enhance p90k secretion and that CD82, acting as a metastasis suppressor, would suppress p90K. However, loss of Vangl1 led to increased p90k secretion [192]. The effect of CD82 on p90K was not investigated.

A second protein detected in the same two hybrid screen, protein kinase C interacting protein (PKCI), binds the intracellular domain of Vangl1 [192]. PKCI/Hint1, a histidine triad protein, interacts with pontin and reptin, which together inhibit β-catenin transcriptional activity [195]. This could explain the putative tumor suppressor activity of PKCI. These findings are intriguing with regard to the role of the reptin/β-catenin complex in expression of CD82 itself. Activation of the reptin/β-catenin complex, due to Wnt activation, suppresses CD82 expression in tumor cells, and the presence of PKCI could potentially prevent CD82 loss. The effects of Vangl1 and CD82 on PKCI function needs to be further evaluated.

2.8. Heterotypic cell–cell interactions

Entry into and out of the blood or lymphatic system requires physical interactions between the tumor cells and the endothelial cells. Thus in addition to mediating cell–cell interactions between tumor cells, CD82 could also impact heterotypic cell–cell interactions.

Several endothelial tetraspanins, including CD9, CD81, and CD151, were found to localize at tumor cell-endothelial cell junctions in both 2D and 3D cultures. Anti-CD9 antibodies specifically inhibited transendothelial migration of melanoma cells [196]. The specific molecules involved in cell–cell interactions were not identified. Tumor-specific expression of α4β1 integrin and binding to VCAM on endothelial cells, a process normally used by activated lymphocytes to migrate to distant tissues, is one possible mechanism. α4β1 integrin can be found on tumor cells, and is known to be regulated by tetraspanins. Whether tetraspanins are required in both cell types for these interactions still needs to be addressed. A recent study in CD151 null mice demonstrated the potential necessity of endothelial-specific CD151 expression for efficient extravasation of tumor cells from the blood stream (Martin Hemler, personal communication). However, blocking CD151 function in tumor cells themselves inhibits the initial dissemination of cells from the primary tumor, but has no effect on entry or exit from the blood stream [136].

The role of CD82 in the specific steps of metastasis has yet to be completely defined. However, tail vein injection of tumor cells re-expressing CD82 does not affect the ability of CD82 to suppress metastasis [5,9], suggesting that CD82's suppressive effects are not limited to events within the primary tumor. Re-expression of CD82 in HT1080 cells or loss of the CD82-specific E3 ubiquitin ligase gp78, which leads to enhanced CD82 expression, did not alter the ability of tumor cells injected via the tail vein to arrive in the lungs, i.e. CD82 had no effect on extravasation [9].

The blood group Duffy antigen (gp-Fy)/receptor for chemokines (DARC) is primarily expressed on the surface of erythrocytes and endothelial cells. DARC is a highly promiscuous chemokine receptor that binds a subset of chemokines involved in pro-angiogenesis, specifically CXCL1-5, CXCL7-11, and CCL2, CCL5, CCL7, and CCL17 [197]. However, chemokine binding does not transduce signals. It has been proposed that DARC serves either as a sink for excess chemokines or in some way regulates chemokine presentation or delivery. Genetic studies in mice suggest DARC exerts a negative effect on pro-angiogenic chemokine function. Transgenic mice over expressing DARC in the endothelium display a reduced angiogenic response in the corneal micropocket assay [198]. Conversely, DARC-deficient mice display enhanced angiogenesis in the matrigel plug assay [197]. Prostate tumors produced in DARC null mice had higher levels of angiogenic chemokines, increased tumor vessel density, and greatly augmented prostate tumor growth compared to tumors in wild type mice [199].

In a yeast two hybrid screen DARC was recently shown to interact with full length CD82. Re-expression of CD82 in tumor cells facilitated their adhesion to DARC positive cells. Adhesion of CD82 positive

tumor cells to DARC positive cells inhibited cell proliferation, reduced cell survival and induced expression of senescence markers TBX2 and p21 in the tumor cells. Furthermore, the ability of CD82 to suppress metastasis was significantly compromised in DARC null mice compared to wild type mice [200]. These findings suggest that CD82 suppresses metastasis by limiting tumor cell viability when cells are shed into the blood stream through interactions with DARC antigen on endothelial and blood cells. If this is true then, this is the first metastasis suppressor shown to act at this specific step in metastasis.

However, there are still several questions to be addressed regarding the putative interaction between CD82 on tumor cells and DARC on endothelial cells. First, it has not been demonstrated that the interaction between CD82 and DARC on these two cell types is direct. CD82 expression alone increases cell–cell interactions between CD82 expressing cells, and endothelial cells express ample CD82. The nature of the CD82-induced cell–cell interaction has not been determined and is not likely to be solely mediated by DARC, since CD82 expression stimulates cell–cell association between cells not expressing DARC. Second, the nature of growth suppressive/senescence signal that is transduced to the tumor cells is unknown. Is this a universal mechanism for cell clearance? If so, why are endothelial cells (which express CD82) not induced to die when then come in contact with red blood cells expressing DARC? Third, it is possible that interactions between CD82 positive tumor cells and endothelial cells also induce endothelial cell death, which could suppress metastasis by limiting efficient endothelial cell recruitment. Finally, can the failure of CD82 to suppress metastasis in DARC null mice be attributed to DARC's ability to modulate chemokine levels rather than a direct effect on tumor cell survival? Chemokines have been implicated in tumor cell homing and survival during metastasis. It is possible that the elevated levels of chemokines that would be expected to occur in the DARC null mice are sufficient to enhance the tumor survival or microenvironment such that metastasis is favored despite the presence of CD82.

Nonetheless, the putative role of CD82/DARC interactions in tumor metastasis is intriguing, and may be important in African-Americans. In addition to its role as a chemokine sink, DARC is the erythrocyte receptor for the malarial parasite. Approximately 70% of African-Americans lack erythrocyte-specific expression of DARC as protection against malaria infection. Coincidently, African-American men have a 60% greater incidence of prostate cancer, which develops as a more aggressive disease (i.e. metastatic) and at younger ages [199]. Thus the putative role of DARC in cancer progression and metastasis should not be overlooked, and that CD82 might be part of the mechanism warrants more intense investigation.

2.9. Survival

An important strategy for successful metastasis is acquiring the ability to survive in multiple environments. Loss of cell adhesion to the ECM is known to induce cell death. Tumor cell detachment and movement away from the primary tumor and transit through the blood stream requires integrin-independent survival. One potential result of CD82 loss would be to enhance tumor survival, through regulation of integrins or other cell surface proteins. CD82, along with tetraspanins CD9, CD53 and Net-6, have all been reported to regulate cell survival.

High levels of CD82 or CD9 expression in CHO cells under conditions that efficiently glycosylate CD82, promoted massive cell death after 11 day s in culture [201]. Cells incapable of glycosylating CD82 did not die, indicating that CD82-induced cell death requires glycosylation—which primarily occurs in the EC2 domain. However, in another study, a C-terminal deletion mutant lacking both the cytoplasmic tail and the EC2 domain was still capable of inducing apoptosis in HeLa cells [202]. The relative expression levels and cell surface expression of these mutants was not reported. It is important that the structure of tetraspanins be preserved for assessing full functionality. For instance, multiple myeloma cell lines over expressing GFP-N-terminal CD82 or CD81, but not C-terminally fused proteins, underwent apoptosis [203]. In fact, the N-terminal tagged constructs failed to express properly at the cell surface. This may have triggered a misfolded protein stress response unrelated to CD82 function. Chimeric tetraspanin molecules, where different domains have been swapped between different tetraspanin molecules, have been used effectively in the past to assess the function of specific tetraspanin domains [107,108,122,204,205].

The tetraspanin Net-6 (Tspan13) is a newly described tumor suppressor gene whose expression level is lowest in highly aggressive breast cancers. Ectopic expression of GFP-NET-6 in MDA-MB-231 partially suppressed proliferative activity in vivo and in vitro, which resulted from an increase in apoptosis [137]. Deletion of Bap31, an ER membrane protein that regulates the export of integral membrane proteins out of the ER, results in decreased cell surface expression of CD9 and CD81. Bap31 did not influence cell surface expression of $\alpha5\beta1$ or $\alpha v\beta3$, but these integrins were not able to maintain ECM adhesion in the absence of growth factors. Subsequently, Bap31 null cells underwent apoptosis. Thus physical separation of tetraspanins from integrins profoundly affects integrin function. It remains to be determined if loss of Bap31 also affects surface expression of other proteins important in tetraspanin/integrin association and whether surface expression of tetraspanins is sufficient to restore integrin function [206].

A few studies suggest some possible mechanisms for tetraspanin-induced death. A monoclonal antibody to CD9 induced TUNEL and annexin-V staining in MKN-28 tumor cells. Apoptotic death was due to antibody-induced tyrosine phosphorylation of p46 Shc, which enhanced JNK and p38MAPK signaling and activated caspases [207]. CD82 was identified in a screen for apoptosis-inducing genes. Forty-two hours after transient transfection of several tumor cell lines (HEK293, PC3, HeLa; MCF7), a 40% increase in the sub G1 population was detected. In these cells CD82 promoted the generation of reactive oxygen intermediates, which upon blockade reversed the apoptotic phenotype. CD82 caused release of the intracellular glutathione (GSH) into the medium and apoptosis could be prevented by the addition of exogenous membrane-permeable GSH. CD82-induced GSH release was mediated by a CD82-dependent increase in Cdc42 activity. Blocking cdc42 activity reversed GSH release and apoptosis [202]. In contrast to CD82, CD53 is dramatically up-regulated in response to radiation exposure and appears to enhance cell survival by inhibiting the intrinsic apoptosis pathway [208]. Expression of CD53 leads to increased intracellular levels of GSH [209].

The ability of CD82 and CD53 to regulate GSH levels and affect cell survival may be related to their association with γ-glutamyl transpeptidase (GGT) [210]. GGT is a membrane protein involved in the extracellular degradation of GSH-conjugates and recycling of extracellular glutathione back into the cell. GGT breaks down extracellular GSH into glutamate and cysteinyl-glycine. Cysteine and glycine are released by a constitutive dipeptidase and all three are transported back into the cell for resynthesis of GSH via γ-glutamylcysteine synthetase (GCS) and GSH synthetase [211,212]. Extracellular GSH can arise from three possible sources in the body: GSH ingested as part of the diet, intracellular GSH transported out of the cell and into the extracellular environment or blood, and GSH secreted into lumens and extracellular spaces by secretory epithelia, such as proximal tubules of the kidney, seminal vesicle, ciliary body epithelium, hepatocytes, and choroid plexus epithelium. Interestingly, many of these specific epithelia and endothelial cells lining the blood vessels are the tissues in which CD82 is highly expressed. Thus one possible function of CD82 is to regulate removal of oxidized GSH-conjugates.

Oxidative stress has been shown to increase the levels of GGT, as a protective mechanism to maintain high cellular GSH levels and prevent apoptosis [211,213]. In immune cells, the GGT anti-apoptotic

survival mechanism appears to involve activation of NF-kB [214]. Many tumor cells display elevated levels of GGT, which appears to be stimulated by Ras signaling. The elevation in GGT would counteract the extreme oxidative tumor environment and favor tumor cell survival by maintaining a more reducing environment. Elevated levels of GGT would also assist in detoxification of chemotherapeutic drugs. Thus GGT could be a potent tumor survival mechanism in vivo.

Extracellular signal transduction pathways have been shown to locally increase ROS, which is required for cell motility. At the same time these same signaling pathways also protect against high levels of ROS, to prevent cell stress and apoptosis; and do so by induction of new GSH synthesis [215–217]. New GSH synthesis is dependent on influx of the necessary components from outside the cells by GGT. Thus tetraspanin regulation of local GSH levels may be required to maintain cellular homeostasis during normal signaling processes. Whether tetraspanins positively or negatively regulate GGT activity or expression has not been determined. However, based on the observed biological responses, the protective effects conferred by CD53 suggest it would enhance GGT activity. Meanwhile, loss of CD82 – as occurs in metastasis – would appear to favor cell survival and increased motility by allowing more efficient GGT activation. Thus CD82 may normally function to limit GGT activity (Fig. 2B).

Re-expression of CD82 in HT1080 cells was shown to reduce tumor cell survival in the lungs 6 h after tail vein injection [9]. The relatively rapid loss in cell viability, within 6 h of entry into the lungs suggests a very rapid onset of cell death. Alternatively, the cells may have already been induced to die prior to entry into the lungs, during their transit through the blood stream, either through interactions with DARC positive cells or due to loss of cell adhesion. Whatever the mechanism, all these studies strongly suggest that one of CD82's primary functions is to regulate cell survival, be it in the primary tumor, the blood stream, or the colonizing organs. The primary function of CD82 is not to induce cell death per se, since many cell types express CD82 under normal conditions. Rather it may be that CD82 sensitizes cells to extracellular cues that warn the cell that the incorrect microenvironment has been detected, i.e. lack of or incorrect cell–ECM or cell–cell interactions are present. If this is true, then CD82 re-expressing tumor cells ought to be more sensitive to death-inducing agents. In one study stable cell lines expressing CD82 were shown to be more sensitive to apoptosis induced by cycloheximide, but not TNFα [202]. The mechanisms involved in CD82-sensitization needs to be investigated to determine whether any of the cell membrane-interacting partners of CD82 are involved.

2.10. Relationship of CD82 to other metastasis suppressors

One question that remains to be answered about CD82, and indeed about any metastasis suppressor, is whether loss of a single metastasis suppressor gene in a primary tumor is sufficient for metastasis. Are these genes global regulators to the extent that loss of a single gene would be sufficient to initiate a metastatic cascade in primary tumors? The xenograft models indicate that restoration of a single gene is sufficient to suppress metastasis, suggesting that blocking one step is sufficient to alter the final outcome. This may reflect the complex nature of the metastatic process; many steps are involved and disruption of one is sufficient to abort the process. Alternatively, because of the relationships that may exist between the different suppressors, the activity of one may affect the activity of another. Is there evidence of functional links between the different metastasis suppressors and CD82? Is there redundancy where loss of other suppressors can substitute for loss of CD82? A few potential relationships are presented below and diagramed in Fig. 3.

2.10.1. Cell–ECM adhesion

nm23 is a kinase that phosphorylates Ksr and reduces MAPK activity. Re-expression of nm23 reduces expression of genes linked to metastasis, such as c-Met, smoothened, frizzled, and MMP2. At least three of these genes, c-Met, frizzled, and MMP2, are potentially impacted by CD82. Two scenarios are possible. Loss of nm23 is sufficient to induce metastasis by simply up regulating c-Met, frizzled, and MMP2 expression. Alternatively, these genes need to be up-regulated, but the suppressive effects of CD82 on their function must also be removed.

KiSS, is a secreted and processed ligand for GPR54 and is a negative regulator of trophoblast migration and invasion [218]. CD82 was

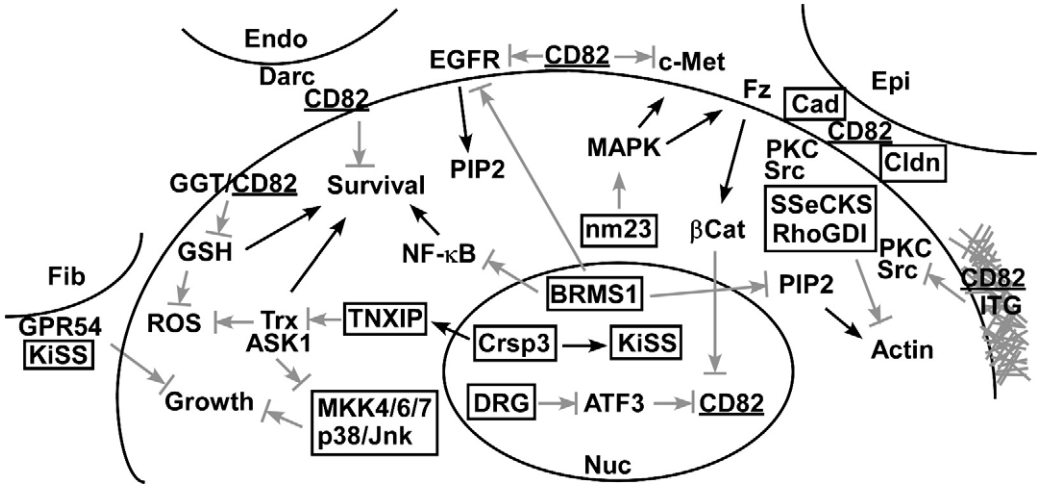

Fig. 3. Molecular relationships between CD82 and other metastasis suppressors. CD82 expression (underline) is potentially controlled by two different suppressors (boxed words). In the nucleus (Nuc) of the tumor cell DRG suppresses the transcription factor ATF3, which negatively regulates *CD82* expression. Enhanced Wnt signaling through frizzled (Fz) to β-catenin (βCat) represses CD82 expression. But Fz expression is in turn suppressed by nm23 via inhibition of MAPK signaling. Nm23 also suppresses c-Met expression. BRMS1 reduces EGFR expression with a concomitant decrease in PIP2 levels, which limits actin reorganization. c-Met and EGFR activation are also suppressed by CD82, which may also contribute to decreased PIP2 levels. CD82 interactions with integrins (ITG) at the matrix, or cadherins (Cad) and claudins (Cldn) at epithelial cell junctions (Epi) stabilizes and limits actin reorganization through suppression of PKC/Src/Rho GTPase signaling which may involve RhoGDI or SSeCKS. CD82 expression increases the sensitivity of cells to death signals through interactions with Darc antigen on endothelial cells (Endo) and suppression of GSH synthesis via GGT. Cell survival signaling is also compromised by BRMS1-mediated reduction in NF-κB signaling. The transcription factor Crsp3 induces two metastasis suppressors, KiSS and TNXIP. TNXIP limits survival via inhibition of the redox protein Trx, but also suppresses cell growth by triggering the MAPK stress response (MKK4/6/7 and p38/Jnk). Simultaneously Crsp3-mediated induction of KiSS and its subsequent binding to GPR54 in surrounding fibroblasts (Fib) triggers a growth suppressive signal to the tumor cell which probably cooperates with the MAPK stress pathway. Thus loss of metastasis suppressors enhances cell signaling leading to enhanced integrin-based motility, loosening of cell–cell junctions, increased cell survival, and ability to grow under stress conditions. All of which are required for successful metastasis.

recently shown to positively control the expression of a protein in decidual cells involved in limiting trophoblast invasion at the maternal–fetal interface [33]. In metastatic cells KiSS binds its receptor on fibroblasts rather than the tumor cells to indirectly suppress tumor cell growth at the site of colonization [219]. CD82 prevents survival of the growth suppressed cells, so KiSS would function downstream of CD82 in a metastatic suppressor cascade.

RhoGDIs sequester Rho GTPases away from their effectors, keeping Rho in an inactive state. RhoGDI2 suppresses metastasis but not tumorigenicity [220]. Active Rho GTPases are critical for cell migration and are regulated by integrin signaling. Direct evidence that CD82 is involved in regulating Rho GTPases in tumor cells has not yet been investigated; however, CD82 and several tetraspanins are known to affect Rho signaling in immune cells. Thus, one possibility is that CD82 facilitates inhibition of Rho GTPases via RhoGDI.

Src-suppressed C kinase substrate (SSeCKS) is a PKC substrate that is negatively regulated by Src. The reported metastasis suppressor functions of SSeCKS strongly resemble those reported for CD82. Re-expression of SSeCKS in tumor cells increases cell–cell adhesion [221], blocks Src-dependent matrigel invasion via inhibition of Rho GTPases [222], and inhibits angiogenesis [223]. The relationship to PKC signaling to SSeCKS and suppression of metastasis has not been investigated. Nonetheless, the strong links between the signaling pathways regulated by CD82 and SSeCKS and the shared biological responses suggests the possibility of interaction between these two metastasis suppressors.

2.10.2. Cell–Cell adhesion

The potential relationships between two metastasis suppressors involved in homotypic cell–cell interactions, Claudin and E-cadherin, were discussed earlier in section 2.7.

2.10.3. Survival

BRMS1 is a transcriptional repressor that suppresses EGFR expression, PIP2 production, and NF-kB transcriptional activity. Its loss promotes adhesion independent survival. It has no impact on the expression of other metastasis suppressors, nm23, CD82, KiSS or E-cadherin. [48,219,224].

The metastasis suppressor Crsp3 is a transcription factor that regulates the expression of two other metastasis suppressors, KiSS and TNXIP. TNXIP/VDUP1 binds the redox-active site of thioredoxin (Trx) to negatively regulate its activity [225]. The Trx system, like the GSH system, acts to reduce intracellular ROS. Trx associates with ASK1, a stress response MAPK kinase. Upon Trx oxidation it dissociates from ASK1 which triggers ASK1 activation and downstream signaling to Jnk/p38 via MKK4/6/7 [226]. Thus loss of TNXIP would enhance the ability of Trx to reduce ROS and maintain cell survival. No links between this pathway and tetraspanins have been reported. The loss of TNXIP may represent an alternative survival mechanism for metastatic cells. Whether there are interactions between Trx and the GSH system with respect to metastasis remains to be established.

DRG1 is nuclear protein whose expression is lost when cells lose PTEN [227]. Re-expression of DRG1 in tumor cells results in a marked decrease in expression of the ATF3 transcription factor. Interestingly, ATF3 binding sites were detected in the CD82 promoter by ChIP analysis (Watabe, personal communication). Tumor samples missing DRG1 also had no CD82, but ATF3 was elevated and vice versa. Thus loss of PTEN, a common occurrence in many tumors and an event associated with progressive disease, would result in loss of DRG1, increased ATF3 and subsequent loss of CD82.

ATF3 is up-regulated in response to stress signaling via Jnk and p38 signaling pathways [228,229]. Stress induced Jnk/p38 signaling due to the tumor microenvironment may also promote ATF3 expression and enhance the loss of CD82. However, elevated ATF3 is also associated with increased apoptosis. Does the loss of CD82 help to diminish the potential apoptotic response?

2.11. Summary

The identification of metastasis suppressor genes and their protein products specifically involved in the complex events associated with the onset and progression of metastatic cancer are providing major insights into the mechanisms of metastasis. The molecular mechanisms by which each of these genes limits metastasis still remain to be fully elucidated. Several specific cellular processes need to be targeted for effective metastasis, and include detachment, motility, invasion, cell survival, and re-growth at the metastatic site. Our current understanding of CD82 function indicates it is likely to be involved in detachment, motility/invasion, and cell survival. As the exact signaling events associated with loss of CD82 and the other suppressor genes become known, as well as the relationship of the signaling pathways to the cellular processes they control, a more comprehensive understanding of which signaling pathways are involved in metastasis becomes evident. With this knowledge comes the ability to design targeted therapies as well as diagnostic and prognostic tests for metastatic cancer.

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REPORT OF INVENTIONS AND SUBCONTRACTS

(Pursuant to "Patent Rights" Contract Clause) (See Instructions on back)

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PLEASE DO NOT RETURN YOUR COMPLETED FORM TO THE ABOVE ORGANIZATION. RETURN COMPLETED FORM TO THE CONTRACTING OFFICER.

1.a. NAME OF CONTRACTOR/SUBCONTRACTOR	c. CONTRACT NUMBER	2.a. NAME OF GOVERNMENT PRIME CONTRACTOR	c. CONTRACT NUMBER	3. TYPE OF REPORT *(X one)*
Van Andel Research Institute	Same as 2.c.	USAMRMC	W81XWH-08-1-0053	X a. INTERIM ☐ b. FINAL

b. ADDRESS *(Include ZIP Code)*	d. AWARD DATE *(YYYYMMDD)*	b. ADDRESS *(Include ZIP Code)*	d. AWARD DATE *(YYYYMMDD)*	4. REPORTING PERIOD *(YYYYMMDD)*
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SECTION I - SUBJECT INVENTIONS

5. "SUBJECT INVENTIONS" REQUIRED TO BE REPORTED BY CONTRACTOR/SUBCONTRACTOR *(If "None," so state)*

NAME(S) OF INVENTOR(S) *(Last, First, Middle Initial)* a.	TITLE OF INVENTION(S) b.	DISCLOSURE NUMBER, PATENT APPLICATION SERIAL NUMBER OR PATENT NUMBER c.	ELECTION TO FILE PATENT APPLICATIONS *(X)* d.				CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER *(X)* e.	
			(1) UNITED STATES		(2) FOREIGN			
			(a) YES	(b) NO	(a) YES	(b) NO	(a) YES	(b) NO
None								

f. EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR/SUBCONTRACTOR		g. ELECTED FOREIGN COUNTRIES IN WHICH A PATENT APPLICATION WILL BE FILED	
(1) (a) NAME OF INVENTOR *(Last, First, Middle Initial)*	(2) (a) NAME OF INVENTOR *(Last, First, Middle Initial)*	(1) TITLE OF INVENTION	(2) FOREIGN COUNTRIES OF PATENT APPLICATION
(b) NAME OF EMPLOYER	(b) NAME OF EMPLOYER		
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SECTION II - SUBCONTRACTS *(Containing a "Patent Rights" clause)*

6. SUBCONTRACTS AWARDED BY CONTRACTOR/SUBCONTRACTOR *(If "None," so state)*

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			(1) CLAUSE NUMBER	(2) DATE *(YYYYMM)*		(1) AWARD	(2) ESTIMATED COMPLETION
None							

SECTION III - CERTIFICATION

7. CERTIFICATION OF REPORT BY CONTRACTOR/SUBCONTRACTOR *(Not required if: (X as appropriate))* ☐ SMALL BUSINESS or X NONPROFIT ORGANIZATION

I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported.

a. NAME OF AUTHORIZED CONTRACTOR/SUBCONTRACTOR OFFICIAL *(Last, First, Middle Initial)*	b. TITLE	c. SIGNATURE	d. DATE SIGNED